Annual Meeting, Central Society for Clinical Research
November 6 and 7, 1959, Drake Hotel, Chicago, Illinois

The Journal of LABORATORY *and* CLINICAL MEDICINE

VOL. 54 SEPTEMBER, 1959 NO. 3

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Clinical and Experimental

Studies on the Stimulation of Hemopoiesis by Batyl Alcohol. James W. Linman, M.D., Martha J. Long, M.S., Donald R. Korst, M.D., and Frank H. Bethell, M.D., Chicago, Ill., and Ann Arbor, Mich. ---------- 335

The Concentration of Serotonin in Normal Human Serum as Determined by an Improved Method. Richard B. Davis, M.D., Minneapolis, Minn. ---------- 344

Serum Phospholipids in Coronary Artery Disease. Louis Cohen, M.D., Chicago, Ill. ... 352

The Base Binding Property of the Serum Proteins With Respect to Magnesium. Ananda S. Prasad, M.B., Ph.D., Edmund B. Flink, M.D., Ph.D., and Horace H. Zinneman, M.D., Minneapolis, Minn. ---------- 357

The Effect of Experimental Hepatic Necrosis on the Vitamin B_{12} Content in Serum and Liver. Nobuo Kato, M.D., and Syuji Murakami, M.D., Nagoya, Japan ---------- 365

Studies of Acid Mucopolysaccharide Metabolism in Human Subjects Given Radioactive Sulfate Intravenously. Clayton Rich, M.D., New York, N. Y. ---------- 381

Sodium Excretion in Renal Disease. A. E. Parrish, N. C. Kramer, M. F. Watt, and J. S. Howe, Washington, D. C. ---------- 387

Renal Function in Human Pregnancy. II. Effects of Venous Pooling on Renal Hemodynamics and Water, Electrolyte, and Aldosterone Excretion During Normal Gestation. N. S. Assali, M.D., W. J. Dignam, M.D., and K. Dasgupta, M.D., Los Angeles, Calif. With the Technical Assistance of M. Ross and L. Friedman ---- 394

Comparison of the Sensitivity of Several Mammalian Cell Types for the Isolation of Poliomyelitis Viruses From Man. David L. Chadwick, M.D., Hartwell H. Welsh, M.S., and Edwin H. Lennette, M.D., Ph.D., Berkeley, Calif. ---------- 409

A Comparative Study of Fetal and Maternal Serum Enzyme Levels. Bernard Lapan, M.D., and Max M. Friedman, Ph.D., New York, N. Y. With the Technical Assistance of Thomas H. Taylor ---------- 417

Serum Turbidity Following a Fat Meal as a Test of Malabsorption. J. D. Kabler, M.D., William H. Atwood, Jr., M.D., and Robert F. Schilling, M.D., Madison, Wis. ---------- 427

(Contents continued on inside front cover)

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Contents—*(Continued from front cover)*

Delay of Absorption of Radiolabeled Cyanocobalamin in the Intestinal Wall in the Presence of Intrinsic Factor. Alfred Doscherholmen, M.D., and Paul S. Hagen, M.D., Minneapolis, Minn. With the Technical Assistance of Lois Olin, B.S. ---- 434

Glomerular Perfusion During Acute Renal Insufficiency From Mercury Poisoning in the Rat. Ethan A. H. Sims, M.D., Herbert I. Goldberg, M.D., Joseph R. Kelly, M.D., and Burton Sisco, B.S., M.S., Burlington, Vt. ---------- 440

Erythrocyte Glucose-6-Phosphate Dehydrogenase Activity and Methemoglobin Reduction. Jean D. Ross, M.D., and Jane F. Desforges, M.D., Boston, Mass. ---------- 450

Aconitase in Human Blood. Ernest Beutler, M.D., and Mary K. Y. Yeh, B.A., Chicago, Ill. ---------- 456

Laboratory Methods

The Determination of Sulfisoxazole and Sulfadimethoxine in Blood and Plasma. Harold L. Rosenthal, Ph.D., and Lois Jud, M.T.(A.S.C.P.), Rochester, N. Y. ---------- 461

A Technique for Determining Whole Blood Clotting Times in Plastic Tubes. Theodore H. Spaet, M.D., Jose Cintron, and Mona Kropatkin, B.S., New York, N. Y. ---- 467

Rapid Separation and Quantitation of Three Phenolic Estrogens by Paper Chromatography. Charles W. Porter, B.A., M.A., Elizabeth L. Clark, B.S., M.T.(A.S.C.P.), and George E. Block, M.D., M.S.(Surg.), Ann Arbor, Mich. ---------- 471

An Automatic Flow Switch. John L. Decker, M.D., Boston, Mass. ---------- 476

Determination of Glycerol in Blood-Glycerol Mixtures. C. A. Crutchfield, Jr., M.S., and H. A. Sloviter, Ph.D., M.D., Philadelphia, Pa. ---------- 478

A Method for Recovering Large Numbers of Platelets and Leukocytes From Normal Human Blood. Henry F. Rattunde, M.D., and Alfred G. Knudson, Jr., M.D., Ph.D., Duarte, Calif. ---------- 484

Liquid Scintillation Counting of Histamine-C^{14} and Its Metabolites. Gildon N. Beall, M.D., and Paul P. VanArsdel, Jr., M.D., Seattle, Wash. With the Technical Assistance of Sue R. Stevens ---------- 487

The Preservation of Typing Sera at Room Temperature With a Five Per Cent Aqueous Solution of Phenol. Victorino de la Fuente, M.D., Manila, Philippines ---------- 495

Inactivation of Coombs Reagent. T. A. Harper, M.B., Yorkshire, England ---------- 497

(Editorial and Business Communications on page 30)

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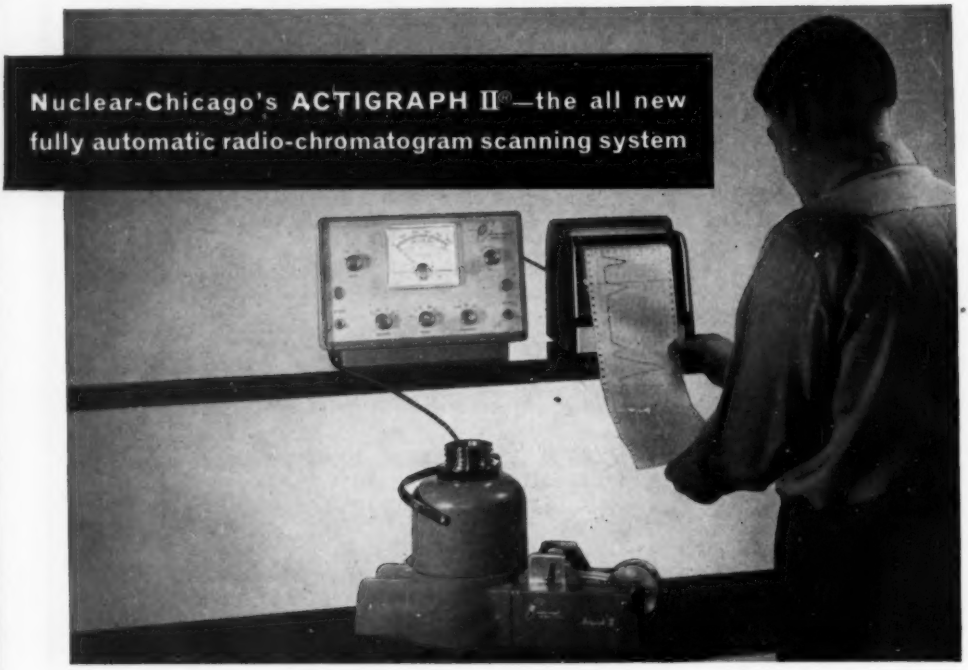

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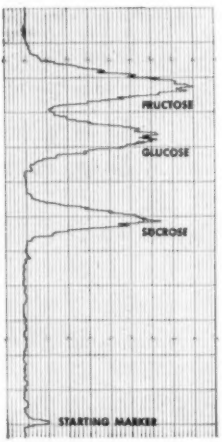

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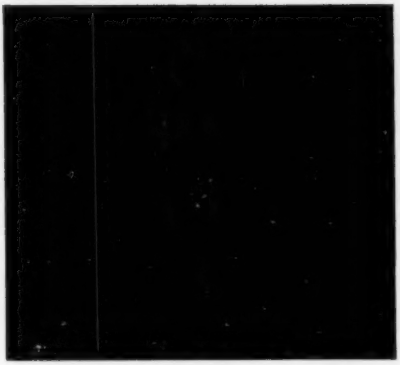

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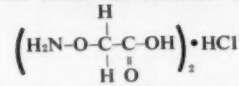

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*'Tis a speculation whether progress in these matters was advanced or retarded by Hans Thacher Clarke's leaving us 32 years ago to build Columbia University's great Department of Biological Chemistry. (Aminooxy)acetic acid was first synthesized in Germany in 1893. It is astounding what a large proportion of all organic chemicals later found interesting for one reason or another were first synthesized in Germany in 1893. Apparently this one, like so many others, just lay there in the literature until Professor Clarke suggested it for reaction with carbonyl compounds. Shortly after the suggestion proved fruitful, he and a collaborator came out with an improved synthesis of the compound. At this point, had he still been heading up the production of Eastman Organic Chemicals, he would doubtless have added it to our list, making it conveniently available to all way back in 1936 and thus advancing progress. But if he hadn't quit to be a college professor, he'd be tied up in a lot of dull business routine. This would have retarded progress because he wouldn't have been sitting around with graduate students and research scholars tossing out brilliant suggestions that are still gaining momentum a quarter century later. Anyway, the synthesis we use for Eastman 5336 now is not identical with Dr. Clarke's. A Texan who tried his synthesis reported she wound up with merely ammonium chloride unless she was very careful.

Cinefluorography

On the other side of town, on November 14 and 15 of last year, there was held the First Annual Symposium on Cinefluorography of the University of Rochester School of Medicine and Dentistry.

To the masses, many of whom may live longer or at least enjoy themselves more than if this conclave had never convened, it would have been mildly interesting to know that x-ray movies of the innards in action had so far advanced from a stunt (for TV science programs) to a diagnostic routine as to make it a subject for a convention. Very mildly interesting, on a November Saturday which already offered the intellectual challenge of picking point spreads for the football games.

To the 150-odd ambitious, clear-eyed radiologists, cardiologists, urologists, internists, laryngologists, and neurosurgeons who came from all over, it was far, far more than a chance to flee the daily hurly-burly for an unequivocally tax-deductible weekend. At this time and place began the process of shaping into standard working technique all the individual improvisations, frustrations, and foolish pioneer feelings experienced late at night in deserted laboratories by medical men coping with electronics, optics, photographic sensitometry, and random fluctuations at the quantum level.

To certain elements of the electronics industry the symposium brought visions of a well-bred market for advanced merchandise, quite free of the fearful dependence on military spending and of the fearful perils of appliance merchandising. If the electronics boys in 1953 hadn't brought out image amplifiers that multiply fluoroscope image intensity a thousandfold or more so that film can be exposed without frying patients, there would have been no symposium. Their emissaries sat unabashed among the M.D.'s.

To the University of Rochester it was a chance to become the national cinefluorographic capital. It had become involved with the subject as early as 1929. To have one member of its radiology department such an expert on motion picture quality that he had produced Poe's "The Fall of the House of Usher" to supra-professional cinematic standards had helped enormously. We helped, too.

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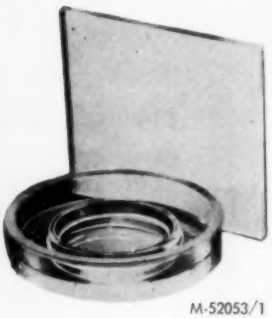

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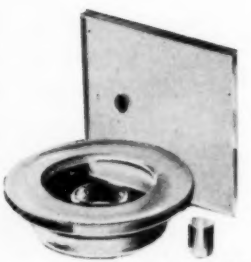

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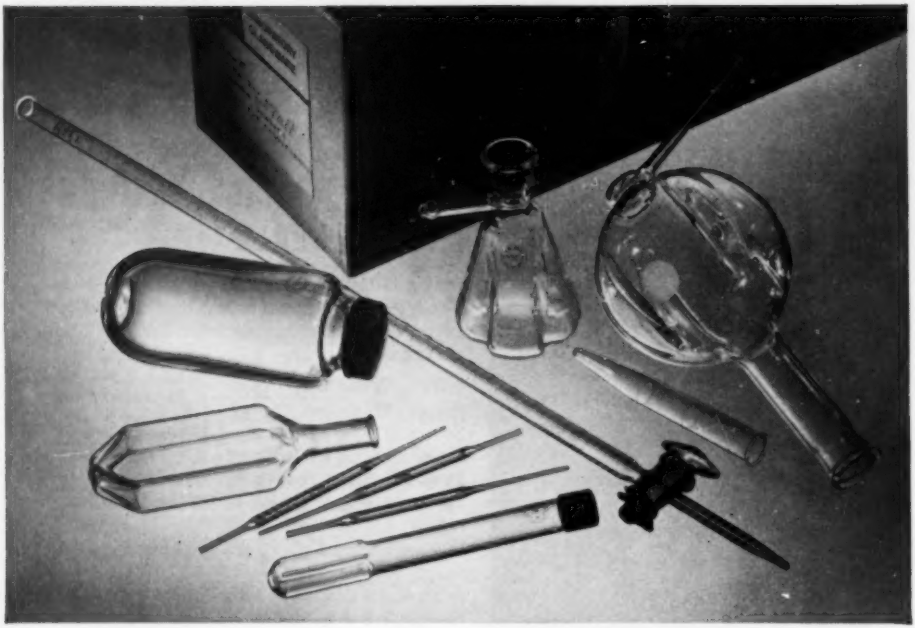

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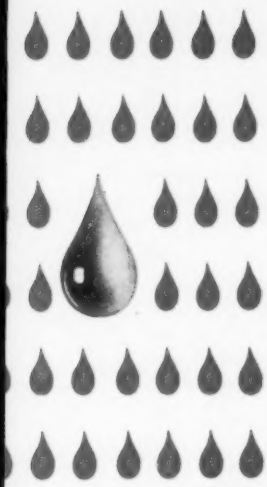

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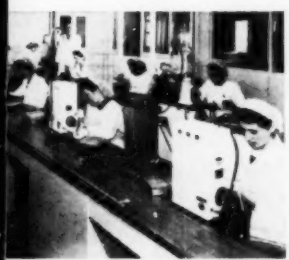

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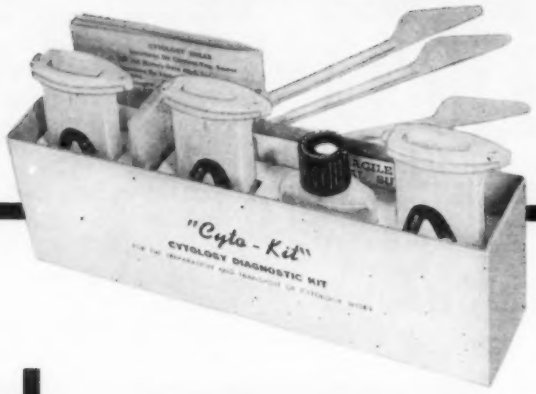

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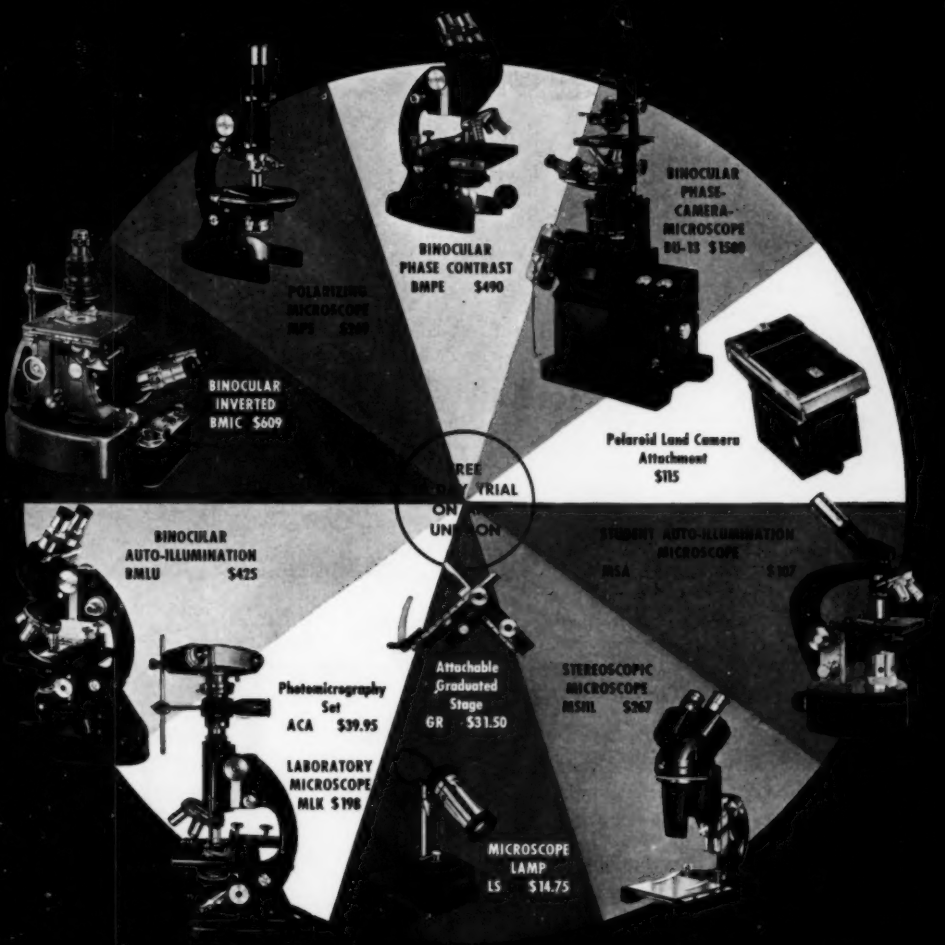
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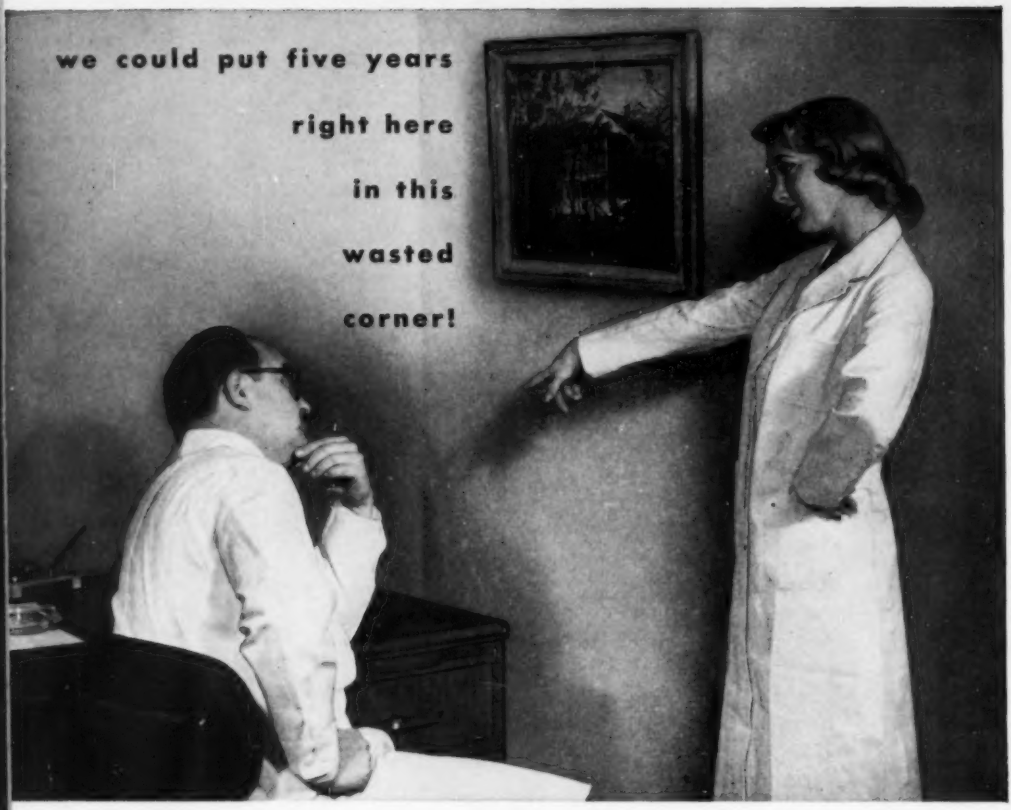

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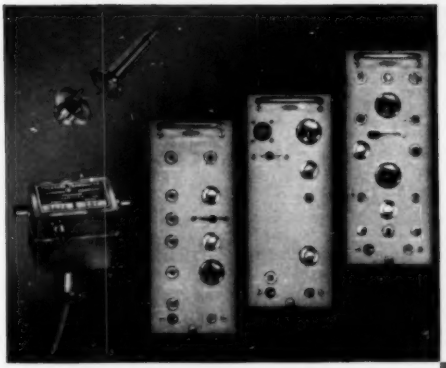

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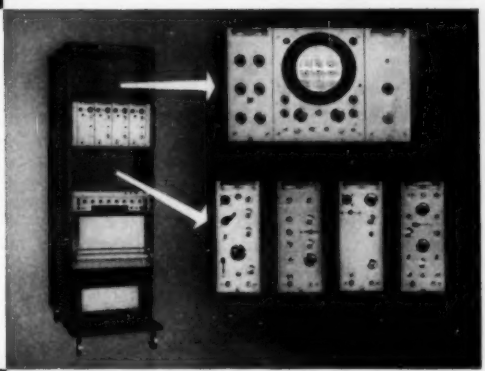

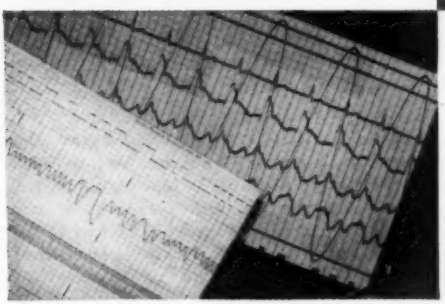

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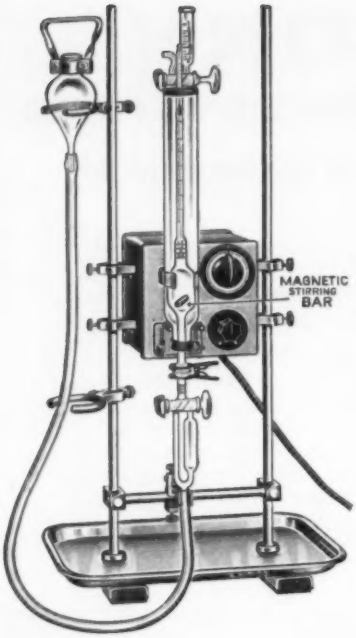

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CLINICAL AND EXPERIMENTAL

STUDIES ON THE STIMULATION OF HEMOPOIESIS BY BATYL ALCOHOL

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BATYL alcohol, the monoglyceerol ether of n-octadecyl alcohol, was reported by Sandler[1] in 1949 to be capable of stimulating erythropoiesis in normal and benzene-treated rats and in normal human subjects. The glyceryl ethers have also been implicated in leukopoiesis and thrombopoiesis,[2-5] and their apparent importance as biologically active substances has been emphasized by discoveries of their wide distribution in nature.[6] Recent studies in our laboratories have confirmed the erythropoietic and thrombopoietic activity of batyl alcohol.[7] Normal rats given daily injections of either 12.5 or 25.0 mg. of batyl alcohol in peanut oil developed erythrocytosis due to the production of microcytes with decreased osmotic resistance, reticulocytosis, and myeloid erythrocytic hyperplasia without associated augmentation in circulating hemoglobin or red cell mass; a response which has otherwise been observed only following the administration of the thermostable, ether-soluble plasma

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erythropoietic factor.[8, 9] Thrombocytosis was also evident in these recipients of batyl alcohol, but they did not manifest consistent or significant increases in their leukocyte counts. It is the purpose of this report to describe observations on the hemopoietic effects of larger doses of batyl alcohol, especially in regard to its granulocytic stimulatory activity.

MATERIALS AND METHODS

Ten normal Wistar strain rats were given 15 daily 50 mg. doses of a 10 per cent suspension in distilled water of racemic batyl alcohol* over a period of 3 weeks, via gastric intubation. A similar number of animals received comparable amounts of distilled water by the same route. Hemoglobins determined by the cyanmethemoglobin technique, microhematocrits, hemacytometer erythrocyte and leukocyte counts, reticulocytes per 1,000 erythrocytes enumerated on dried brilliant cresyl blue coverslip films counterstained with Wright's stain, absolute numbers of the various leukocytic elements derived from 200 cell differential counts, and platelets counted by phase microscopy comprised the hematologic studies performed in these animals. All values were obtained prior to treatment and every 7 days thereafter with the exception of the reticulocytes which were counted twice weekly.

At the end of the 3-week treatment period, 5 rats in each group were killed and femoral marrow was examined utilizing a technique previously described.[10] The remaining animals were observed for an additional 2 weeks to ascertain the rapidity of return to normal hemic equilibrium.

The effect of batyl alcohol on the incorporation of $iron^{59}$ in hemoglobin was studied in each of two groups of 4 rats conditioned by starvation and hypophysectomy, respectively. Following 3 daily 25 mg. injections of batyl alcohol, $iron^{59}$ was given intravenously to these recipients, and the per cent uptake by the circulating erythrocytes was determined 24 hours later. The uptake of $iron^{59}$ by in vitro marrow cultures was used as an additional method[11, 12] to evaluate the erythropoietic activity of batyl alcohol. Normal rat marrow cells were suspended in Eagle's solution (60,000 cells per cubic millimeter) and 0.7 ml. introduced into each of a series of tubes together with 0.3 ml. of normal serum, 0.6 ml. of Eagle's solution, and 0.1 ml. (2 μc) of $iron^{59}$ globulin complex. The total volume in each tube was brought to 2.0 ml. by the addition of 0.3 ml. of either batyl alcohol in saline (25 mg. per milliliter), batyl alcohol in peanut oil (6.25 mg. per milliliter), "anemic" serum, or normal serum. The uptake of $iron^{59}$ by the cells contained in each tube was determined after they had been incubated for 20 hours.

RESULTS

The hematologic responses in normal rats subsequent to orally administered batyl alcohol were characterized by erythrocytosis and reticulocytosis without change in hemoglobins or hematocrits and by thrombocytosis and leukocytosis (Figs. 1 and 2 and Table I). The small cells responsible for the erythrocytosis were easily discerned on stained films and demonstrable graphically by Price-Jones measurements. The leukocytosis in the recipients of batyl alcohol was due chiefly to a relative and absolute neutrophilia (Table II). Lymphocytes, monocytes, and eosinophils were slightly increased but to a much lesser degree than were the neutrophils.

Hyperplasia of erythrocytic and granulocytic myeloid elements was evident at the end of the treatment period in the animals receiving batyl alcohol (Fig. 3), and increased mitotic activity was noted in both of these cell series. Megakaryocytes were also present in greater than normal numbers.

*The synthetic batyl alcohol used in this study was kindly supplied by George A. Breon & Company, New York, N. Y.

All values in the rats not killed for marrow studies returned promptly to normal baseline and control levels after the batyl alcohol was discontinued (Figs. 1 and 2). The explanation for the drop in circulating red cell mass one week after treatment was stopped and the return to normal 7 days later is not apparent. Since it was not accompanied by a corresponding decrease in the hemoglobin level and has not been a consistent observation in other animals given batyl alcohol nor in all rats in this group, it most likely represents a fortuitous finding of doubtful significance.

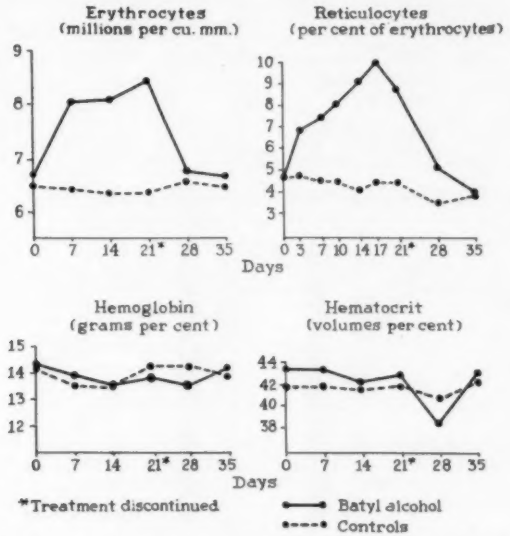

Fig. 1.—Erythrocytosis and reticulocytosis without increases in hemoglobin or hematocrit levels in normal rats given daily 50 mg. doses of batyl alcohol orally. Average determinations of 10 animals in each group.

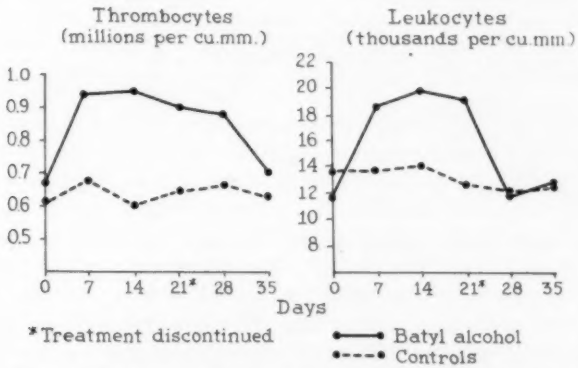

Fig. 2.—Thrombocytosis and leukocytosis in recipients of batyl alcohol. The increase in leukocytes was due largely to a relative and absolute neutrophilia. Average counts of 10 rats in each group.

The determinations in the control group were constant throughout. All animals remained healthy and demonstrated no untoward reactions from the batyl alcohol or repeated gastric intubations.

TABLE I. STANDARD DEVIATIONS IN THE ERYTHROCYTE, THROMBOCYTE, LEUKOCYTE, AND RETICULOCYTE COUNTS

DETERMINATION	GROUP	BASE LINE		3 WEEKS	
		MEAN	S. D.	MEAN	S. D.
R.B.C. × 10^6	Batyl alcohol	6.68	0.48	8.42	0.44
	Controls	6.49	0.33	6.39	0.30
Platelets × 10^6	Batyl alcohol	0.677	0.091	0.896	0.090
	Controls	0.608	0.111	0.646	0.099
W.B.C. × 10^3	Batyl alcohol	11.8	1.4	19.1	2.7
	Controls	13.8	2.2	12.9	2.7
Reticulocytes (%)	Batyl alcohol	4.6	1.5	8.7	1.1
	Controls	4.6	2.0	4.3	0.7

TABLE II. CIRCULATING NUCLEATED CELLS PER CUBIC MILLIMETER (AVERAGE DETERMINATIONS OF 10 RATS IN EACH GROUP)

CELL TYPE	BATYL ALCOHOL		CONTROLS	
	BASE LINE	3 WEEKS	BASE LINE	3 WEEKS
Total leukocytes	11,800	19,100	13,800	12,900
Neutrophils	1,128	5,262	1,704	1,805
Lymphocytes	10,167	13,033	11,525	10,491
Monocytes	323	598	388	283
Eosinophils	160	401	96	137
Basophils	27	26	53	23

TABLE III. FAILURE OF BATYL ALCOHOL (25 MG./DAY FOR 3 DAYS) TO ENHANCE THE INCORPORATION OF $IRON^{59}$ IN HEMOGLOBIN OF RECIPIENT RATS (AVERAGE DETERMINATIONS OF 4 ANIMALS IN EACH GROUP ± 1 S. D.)

TYPE OF RECIPIENTS	PER CENT $IRON^{59}$ R.B.C. UPTAKE AT 24 HOURS		
	NORMAL SERUM	BATYL ALCOHOL	"ANEMIC" SERUM
4-day starved rats	6.6 ± 2.7	6.5 ± 1.6	19.9 ± 7.4
14-day posthypophysectomized rats	2.5 ± 0.5	3.1 ± 2.2	10.9 ± 4.6

TABLE IV. FAILURE OF BATYL ALCOHOL TO ENHANCE THE UPTAKE OF $IRON^{59}$ BY IN VITRO CULTURES OF NORMAL RAT MARROW CELLS (AVERAGE DETERMINATIONS OF 4 TUBES WITH EACH TEST SUBSTANCE ± 1 S. D.)

TEST SUBSTANCE	PER CENT $IRON^{59}$ MARROW CELL UPTAKE AT 20 HOURS
Batyl alcohol in saline	1.4 ± 0.3
Batyl alcohol in peanut oil	0.9 ± 0.2
Normal serum	0.9 ± 0.4
"Anemic" serum	5.8 ± 1.0

Batyl alcohol, which failed to augment the hemoglobin and hematocrit levels of recipient rats (Fig. 1), was also ineffectual in enhancing the incorporation of $iron^{59}$ in hemoglobin. As shown in Table III, the erythrocyte uptake of $iron^{59}$ in rats given batyl alcohol was comparable to that observed in animals injected with normal serum whereas "anemic" serum was unquestionably active. Studies which utilized the uptake of $iron^{59}$ by in vitro cultures of normal rat marrow cells yielded similar data (Table IV).

DISCUSSION

The experimental observations described herein support the theory that batyl alcohol stimulates erythroblastic cellular division in the normal rat without augmenting hemoglobin synthesis. In addition, they confirm its thrombocytosis-promoting effect and granulocytic stimulatory activity. The data suggest that batyl alcohol may also influence other leukocytic elements. Administration by the oral route is shown to be effective. Larger amounts of batyl alcohol are apparently required to evoke leukocytosis in recipient animals than are needed to exert detectable erythropoietic and thrombopoietic activity. This relationship between dosage of batyl alcohol and the type and degree of response[7] probably explains some of the negative results which have been reported.

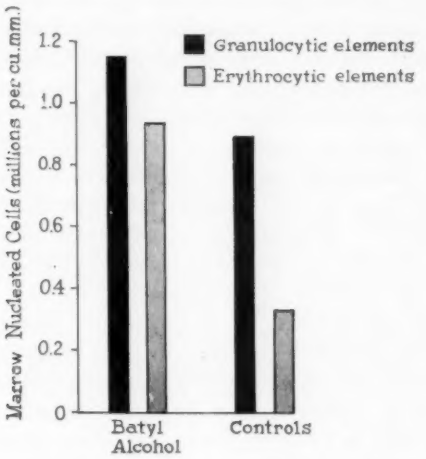

Fig. 3.—Average marrow nucleated cell counts of 5 animals in each group demonstrating both erythrocytic and granulocytic hyperplasia in the rats receiving batyl alcohol.

The magnitude and uniformity of the erythrocyte, reticulocyte, thrombocyte, and leukocyte increases in the recipients of batyl alcohol together with the marrow findings and the rapid return to normal values after the treatment was stopped are such that a causal relationship cannot be denied. The myeloid hypercellularity and the failure to elicit increases in hemoglobins or hematocrits seems to eliminate hemoconcentration as an explanation of the findings. Consequently, primary myelopoietic stimulation or accelerated myelopoiesis due to hemolysis would appear to be the only alternative explanations for the hematologic phenomena observed in normal rats given batyl alcohol.

Since a number of lipid substances are known to exert a hemolytic effect and because thrombocytosis and leukocytosis may be associated with hemolysis, it is necessary to exclude this possibility before assigning true hemopoietic properties to batyl alcohol. The myeloid erythrocytic hyperplasia, reticulocytosis, leukocytosis, thrombocytosis, and stable hemoglobin and hematocrit

values are all compatible with a compensated hemolytic state, but the erythrocytosis is not unless it represents red cell cytoclasis. Such a mechanism with impaired viability of the resultant erythrocytic fragments would explain all of the above findings in addition to the presence of microcytes with decreased osmotic resistance. Under these circumstances, however, it would be necessary to postulate definite augmentation in hemoglobin production as a prerequisite for the maintenance of normal hemoglobin and hematocrit levels, and enhanced incorporation of iron[59] in hemoglobin should have been evident in recipients of batyl alcohol. The reticulocytosis occurs so promptly and is of such a magnitude there can be little doubt but that hemolytic activity of this degree, whether compensated or not, would be reflected by increased erythrocyte iron[59] uptake. For this reason, it would not seem possible to evoke hemolysis as an explanation for the hemopoietic effects of batyl alcohol. Furthermore, hemolysis per se does not adequately explain the thrombocytosis and leukocytosis since their association with hemolytic disease is only an unexplained observation.

On the basis of existent experimental data, therefore, it can be concluded that batyl alcohol exerts a primary stimulatory effect on hemopoiesis. However, many questions remain unanswered as to what role batyl alcohol may play in the maintenance of normal hemic equilibrium or in certain pathologic states. Foremost among these is the apparent inconsistency between the dosage of batyl alcohol needed to alter hemopoiesis in normal rats and the much smaller quantities of a substance possessing such specific biologic activity which would be expected to exert a readily detectable effect. In view of the absorptive difficulties previously encountered,[7] it is conceivable that the exogenous requirements may greatly exceed the endogenous need, or there may exist differences in activity between the racemic synthetic material studied and the optically active natural form. Another quite likely possibility is that batyl alcohol may not be identical with but rather closely related to, or perhaps a precursor of, some other more active substance. Additional studies are in progress in our laboratories and those of other investigators pertaining to the hemopoietic activities of the other glyceryl ethers, selachyl alcohol and chimyl alcohol. Although both batyl and chimyl alcohol are solids, selachyl alcohol, which differs only slightly in chemical structure, is liquid at room temperature and is the glyceryl ether found most abundantly in nature.[13]

The alterations in hemic equilibrium induced by batyl alcohol assume added interest in the light of recent advances in our knowledge of the mechanisms which govern erythropoiesis.[14, 15] Although the erythropoietic stimulatory effect of certain plasmas and sera has been ascribed by many investigators to only one substance which is apparently a mucoprotein,[15-18] our observations indicate that there are two humoral erythropoietic factors.[8, 19-21] The protein factor is relatively thermolabile and ether-insoluble and enhances hemoglobin production as well as erythropoiesis in recipient animals. We are in accord with the theory[15, 22, 23] that this agent most likely augments hemoglobin formation by increasing erythrocytic differentiation from the pluripotential

myeloid reticulum cells or from primitive erythroblasts and that subsequent maturation and hemoglobin synthesis probably occur simultaneously at a fixed rate which appears to be independent of the humoral stimulus or total output by the marrow.[24] It is apparent that an increase in the number of erythrocytic precursors differentiated from the primitive dividing cells will, if other conditions remain unchanged, lead to enhanced hemoglobin production. There is, however, no compelling evidence that the multiplication of myeloid erythrocytic elements, which occurs chiefly if not entirely by homeoplastic mitosis, and the synthesis of hemoglobin must be responsive to a single stimulus. Examples of disparity between these seemingly diverse aspects of erythropoiesis are evident, and our previous studies indicate that the number of cellular divisions which the erythrocytic precursors undergo during maturation may be influenced by a second humoral factor. The latter agent is resistant to heating for prolonged periods at 100° C., is ether-soluble, and appears to be a lipid. It is hypothecated that these two factors control, independently, the number of erythrocytes and the quantity of hemoglobin produced and that their combined activities determine, in addition, the size and hemoglobin content of each red cell.

The chemical and physiologic attributes common to batyl alcohol and the thermostable, ether-soluble, plasma erythropoietic factor suggest, reasonably but as yet without proof, that they are closely related. If this relationship is established by further investigation, the pathogenesis of the leukocyte and thrombocyte increases associated with such conditions as acute hemorrhage, hemolysis, and polycythemia vera may be clarified. The apparent thrombocytosis-promoting effect in normal rats of the ether-soluble fractions of plasmas from patients with polycythemia vera[21] supports this hypothesis. The consistent failure to observe leukocytosis and thrombocytosis in experimental animals or human subjects with erythroid hyperplasia due to stimulation by plasma of anemic or hypoxic donors or to endogenously enhanced humoral factor activity does not necessarily rule against this possibility. It may reflect varying degrees of sensitivity of the different myeloid elements comparable to the dose-response relationship observed in normal rats given batyl alcohol. In this connection it may be noted that there exists a suggestive correlation between leukocytosis, thrombocytosis, and the severity of certain acute hemolytic processes. Furthermore, the granulocyte and thrombocyte increases following acute hemorrhage are related to the rapidity and magnitude of the blood loss and disappear after subsidence of the initial and presumably maximal stimulus to erythropoiesis.

Although the physiologic significance of the hemopoietic effects of batyl alcohol has yet to be elucidated, the phenomena observed are in apparent accord with the theory that all aspects of hemopoiesis are under humoral regulatory control and that a single substance or activator-inhibitor complex may affect, although not to the exclusion of other mechanisms, the formation of all hemic elements. Batyl alcohol or some closely related compound may be a component of such a system.

SUMMARY

1. The hemopoietic effects in normal rats of oral batyl alcohol consist of erythrocytosis, due to the production of microcytes, together with reticulocytosis, thrombocytosis, leukocytosis, and hyperplasia of all myeloid elements.

2. The erythrocytic response is identical with that induced by the thermostable, ether-soluble plasma erythropoietic factor and is not associated with increased hemoglobin or hematocrit levels. Batyl alcohol also fails to enhance the incorporation of iron^{59} in hemoglobin of recipient rats or in vitro bone marrow cultures.

3. Larger amounts of batyl alcohol are required to elicit leukocytosis, which is primarily the result of a relative and absolute neutrophilia, than are needed to exert detectable erythropoietic and thrombopoietic activity.

4. It is suggested that batyl alcohol or some closely related substance may play an important role in the regulatory control of hemopoiesis.

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THE CONCENTRATION OF SEROTONIN IN NORMAL HUMAN SERUM AS DETERMINED BY AN IMPROVED METHOD

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NUMEROUS methods have been employed for the isolation and quantitation of serotonin in serum and blood. Determination in normal human blood has depended on isolation of platelets by differential centrifugation, and measurement of serotonin after its release from platelets. Quantitation of serotonin after isolation has depended (1) on bio-assay using bovine carotid artery muscle strips,[1] rat colon or uterus,[2-7] or the isolated perfused rabbit ear[8] or (2) on spectrophotofluorometry.[9] Concentrations of serotonin have been expressed in terms of 10^9 platelets, milligrams of platelet protein, or milliliters of blood. A summary of the methods employed, with normal human blood and serum values taken from the literature, is presented in Table I.

Because of variable methods of isolation, and well-recognized difficulty in the precise quantitation of platelets,[10, 11] a method was sought which would provide a high degree of reproducibility and which would circumvent platelet isolation by determination of serotonin after its release into serum from blood clotted under standard conditions.

MATERIALS AND METHODS

Serotonin was used as the creatinine sulfate complex (obtained from Nutritional Biochemicals Corporation). Concentrations were expressed in terms of the free base. G. E. Dri-Film was obtained from E. H. Sargent & Company. Medium-sized crystals of ascorbic acid (obtained from Merck & Company) were ground to a fine powder prior to use.

Fluorescence was measured using a Farrand spectrofluorometer, Model A, calibrated according to the method of Sprince and Rowley.[12] A grating monochromator with a spectral range of 220 to 440 mμ and a Corning glass filter No. 9863 between the light source and monochromator were employed for excitation of fluorescence. A grating monochromator with a spectral range of 400 to 700 mμ and a Corning glass filter No. 3389 between the cuvette and monochromator were used to measure fluorescence. Fixed slit widths of 10 mμ were used both for excitation and measurement of fluorescence. A 1 by 1 by 5 cm. quartz cuvette in a specially built adapter was used. Readings were made in microamperes. All glassware was acid washed; butanol and heptane were prepared by washing with 0.1 N NaOH, 0.1 N HCl, and distilled water.[9]

Blood samples were obtained by gentle suction after venipuncture with an 18 gauge needle previously treated with Monocote E.† All syringes were treated with G. E. Dri-Film (methyl chlorosilane) according to the method of Tullis and Rochow.[13] Blood was obtained

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†Obtained from Armour & Company, Chicago 11, Ill.

TABLE I. BLOOD AND SERUM SEROTONIN VALUES IN MAN, AS REPORTED BY VARIOUS INVESTIGATORS

INVESTIGATOR	NUMBER OF SUBJECTS	MATERIAL	METHOD	VALUE
Erspamer[3]	A. 14 young adults B. 16 elderly adults	Serum	Bio-assay (rat uterus)	A. Mean: 0.12 μg/ml. Range: 0.07-0.20 μg/ml. B. Mean: 0.09 μg/ml. Range: 0.03-0.15 μg/ml.
Zucker, Lewis, and Borrelli[26]	29 normal persons	Serum	Bio-assay (isolated perfused rabbit ear)	0.062 μg/ml. ± 0.028 μg/ml.
Sharman and Sullivan[4]	2 normal persons	Serum (acetone extract)	Bio-assay (rat uterus)	0.15, 0.16 μg/ml.
Snow, Lennard-Jones, Curzon, and Stacey[5]	25 persons	Serum (acetone extract)	Bio-assay (rat uterus)	0.07 μg/ml. ± 0.02 μg/ml.
Barkhan[7]	31 persons	Serum	Bio-assay (rat uterus)	Mean: 0.098 μg/ml. Range: 0.044–0.247 μg/ml.
Hardisty and Stacey[6]	35 normal persons	Platelet suspension (acetone extract)	Bio-assay (rat uterus)	0.057 ± 0.018 μg/10^8 platelets (0.16 ± 0.06 μg/ml. of blood)
Humphrey and Jaques[2]	2 "samples"	Platelet suspension	Bio-assay (rat colon, uterus)	0.2, 0.3 μg/10^9 platelets (0.19 μg/ml. of blood)
Zucker and Borrelli[8]	8 young adults	Platelets (saline extract of frozen, dried platelets)	Bio-assay (isolated perfused rabbit ear)	0.76 μg/10^9 platelets; range: 0.5–1.3 μg/10^9 platelets (0.195 μg/ml. blood)
Weiner and Udenfriend[28]	94 hospitalized patients	Platelet suspension	Spectrophotofluorometric	0.87 μg/10^9 platelets; range: 0.025–2.2 μg/10^9 platelets (0.22 μg/ml. blood)
Sjoerdsma, Weissbach, Terry, and Udenfriend[29]	10 normal persons	Platelet suspension	Spectrophotofluorometric	0.26–0.63 μg/mg. platelet protein
Sjoerdsma, Weissbach, and Udenfriend[30]	"about" 40 normal persons	Platelet suspension	Spectrophotofluorometric	0.1–0.3 μg/ml. blood

from healthy young adults approximately 2 hours after eating. Blood specimens also were obtained from 2 hospitalized patients with metastatic carcinoid for the determination of optimum working conditions. Seven milliliters of blood was added to serum tubes containing 41.0 mg. of ascorbic acid and immediately was inverted gently 12 to 14 times using a Parafilm* cover. The blood was allowed to clot by standing for 30 minutes at room

*Marathon Corporation, Menasha, Wis.

temperature (24° C.) and placed in a refrigerator at 4° C. Six hours after the blood was drawn it was centrifuged, and 3.0 ml. of serum was removed for assay.

The analytic method used was a modification of those of Udenfriend and associates[14] and Bogdanski and co-workers.[15] To 3.0 ml. of serum was added 0.12 ml. of 1 N NaOH and 0.38 ml. of 10 per cent $ZnSO_4$. The tube was agitated vigorously and the precipitate centrifuged; 1.5 ml. of supernatant was transferred to a graduated centrifuge tube, and anhydrous Na_2CO_3 was added to adjust the pH approximately to 10. The alkaline solution then was transferred to a 50 ml. glass-stoppered centrifuge tube containing 5 Gm. of NaCl and 5.0 ml. of borate buffer.* The total volume was adjusted to 15 ml. with water from washings of the graduated centrifuge tube. Twenty milliliters of butanol was added and the mixture shaken for 15 minutes. The mixture was transferred to a 40 ml. Pyrex tube, and centrifuged at 3,000 r.p.m. until a clear layer of butanol was obtained. Ten milliliters of butanol was removed, and washed twice by shaking with an equal volume of borate buffer. The buffer was completely aspirated and discarded, then 1.5 ml. of 0.1 N HCl and 20 ml. of n-heptane were added. The mixture was shaken for 5 minutes; 1.5 ml. of the acid layer then was mixed with 0.45 ml. of 12 N HCl, and fluorescence was measured at 550 mμ with excitation at 295 mμ. Centrifugation was carried out routinely at 4° C. In the presence of low amounts of serotonin somewhat greater sensitivity is obtained by using 6.0 ml. of serum with the following volumes of reagents: 0.25 ml. of 1 N NaOH, 0.75 ml. of 10 per cent $ZnSO_4$, a 3.0 ml. aliquot of supernatant from the protein precipitate, 7.5 ml. of butanol removed after centrifugation, and 15 ml. of heptane.

RESULTS

The time of removal of serum, and amounts of ascorbic acid required were varied in order to determine optimum working conditions. Blood was incubated at 37° C. for 15 and 45 minutes with two different amounts of ascorbic acid; the serum then was removed and serotonin was determined. Blood which was allowed to coagulate at room temperature also was refrigerated 5½, 11½, and 23½ hours after removal of serum. The results in two patients with metastatic carcinoid and in two normal persons are shown in Table II.

TABLE II. RELATIONSHIP BETWEEN THE TIME OF REMOVAL OF SERUM FROM THE CLOT AND SEROTONIN CONCENTRATION

	SEROTONIN CONCENTRATION (μg/ML.)				
	30 MG. ASCORBIC ACID		45 MG. ASCORBIC ACID		
		CARCINOID		CARCINOID	
TIME	NORMAL	PT. A.F.	NORMAL	PT. R.H.	PT. A.F.
15 min.	0.15	4.80	0.11	1.25	4.20
45 min.	0.10	3.20	0.10	1.16	2.85
6 hr.	0.18	5.30	0.15	1.26	4.70
12 hr.	0.16	4.90	0.15	1.16	—
24 hr.	0.17	4.60	0.15	1.16	4.45

Blood specimens were obtained from 2 different patients with carcinoid syndrome and from 2 different normal persons. Serum was removed at 15 and 45 minutes after the blood was drawn from samples incubated at 37° C., and at 6, 12, and 24 hours from samples allowed to clot at 24° C. for 30 minutes and then kept at 4° C. for 5½, 11½ and 23½ hours.

In the presence of elevated levels of platelet and plasma serotonin the measured serotonin concentration increased with increasing amounts of ascorbic acid (Fig. 1). When 90 mg. of ascorbic acid or more per 7 ml. of blood was added clotting did not occur, and only a small fraction of the amount obtained

*It has been found that the use of a buffer of pH 11 or more results in a 50 per cent loss of serotonin, hence the pH must be carefully adjusted to 10.[16] See also Reference 17.

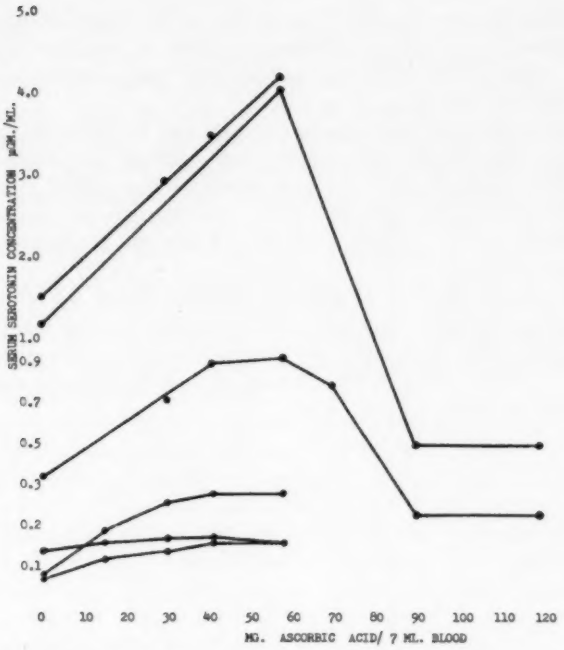

Fig. 1.—Relationship between the amount of ascorbic acid used and the serum serotonin concentration. The upper 3 curves represent 3 separate blood samples from 2 patients with metastatic carcinoid. The lower curves represent determinations on 3 different normal individuals. In every determination the serum was removed 6 hours after the blood was drawn.

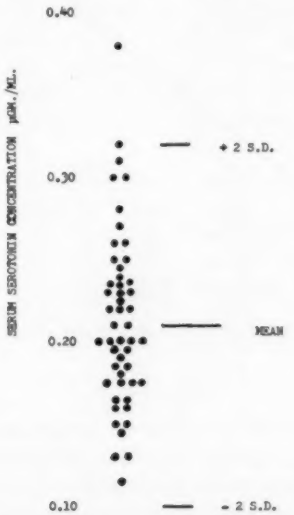

Fig. 2.—Distribution of serum serotonin concentrations in 50 normal persons.

with 58 mg. was found. In normal persons a similar but less pronounced increase of concentration with increasing amounts of ascorbic acid occurred. No increase in the fluorescence of a water blank was observed when 200 mg. of ascorbic acid was added.

Serum serotonin concentrations were measured in duplicate in 50 normal young adults. The mean concentration was 0.21 μg per milliliter, with a range (± 2 S.D.) from 0.10 to 0.32 μg per milliliter (Fig. 2). One determination exceeded 0.32 μg milliliter. There was an average variation between duplicate samples of 0.005 μg per milliliter (2.4 per cent of the mean concentration).

To determine the recovery of serotonin added to blood, 6.0 ml. of blood was added to varying amounts of serotonin in 1.0 ml. of normal saline with 41.0 mg. of ascorbic acid. The total serotonin recovery in the serum plus saline was determined. As seen in Fig. 3, 79 per cent of added serotonin was present in the serum-saline mixture.

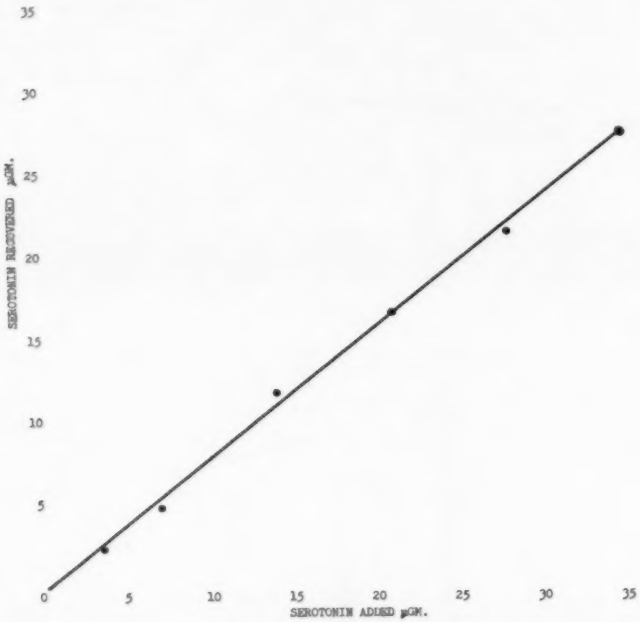

Fig. 3.—Relationship between the total serotonin added to blood and the amount recovered in a serum-saline mixture.

DISCUSSION

Coagulation of blood has been known to release a serum vasoconstrictor substance since 1868, when Ludwig and Schmidt first showed that defibrinated blood contained a substance which produced increased resistance to perfusion of dog muscle.[18] Serotonin is found only in the platelets of normal blood, and is released by mechanical trauma (e.g., freezing and thawing, ultrasonic

vibrations, etc.), contact of platelets with unsiliconized glassware, alkaline tissue extracts,[19] reserpine,[20] antigen-antibody reactions,[21, 22] and bacterial endotoxin.[23] Clotting does not cause serotonin liberation by the mechanical effects of clot formation,[24] but liberation is caused by thrombin formation and coincides with the disappearance of platelet adenosinetriphosphate (ATP).[25]

In the past, serum serotonin concentrations have been recognized to be substantially less than those of whole blood, calculated from the assay of platelet-rich plasma (Table I). Zucker and associates found an average serum serotonin concentration of 0.062 μg per milliliter in 29 persons, and believed that the low recovery was partially attributable to serotonin adsorption on erythrocytes,[26] a belief with which Cargill-Thompson and associates concurred.[17] Hardisty and Stacey[6] found a wide variation of serum serotonin in similarly treated blood specimens, and concluded that only an inconsistant fraction of platelet serotonin reached the serum.[6]

Recently Ling and Blum[27] reported that serotonin incubated with whole blood or crystalline hemoglobin was rapidly destroyed, probably by oxidation. They found that denaturation of hemoglobin at pH 3.0, carbon monoxide, trichloroacetic acid (TCA), and ascorbic acid prevented destruction, but that glutathione did not.[27] These findings suggested that the previously reported low and variable amounts of serotonin in serum were due to serotonin destruction in varying degree, and that if serotonin was measured in serum obtained from blood clotted in the presence of ascorbic acid larger amounts and more reproducible results might be obtained.

Needles treated with Monocote E and silicone-coated syringes were used in order to prevent disruption of platelets and the release of serotonin before the blood could come in contact with ascorbic acid. Blood, added to ascorbic acid, was incubated for 15 and 45 minutes at 37° C. since Zucker and Borrelli[8] found no constant variation in serum serotonin under those conditions in the absence of ascorbic acid. Also, blood was arbitrarily allowed to stand for 6, 12, and 24 hours to determine if early serotonin release, effected by thrombin formation, might be augmented by serotonin release coincident with platelet breakdown.

Maximum amounts of serotonin were found when blood was allowed to stand for 6 hours after being drawn. Lesser concentrations were found in blood incubated for 45 minutes at 37° C. and in blood which was allowed to stand for 12 and 24 hours; this was probably due to gradual serotonin destruction. The variation was small, however, when normal blood was used. With an increased amount of ascorbic acid in normal blood (58 mg. per 7 ml.) no increase in serotonin concentration resulted over the amount found with 41 mg. per 7 ml.

An attempt also was made to find an amount of ascorbic acid at which constant serotonin concentration would be found in carcinoid blood, in the presence of increased amounts of ascorbic acid, as found for normal blood. When 70 mg. of ascorbic acid was used the clot was poorly formed and a lower serotonin concentration was found; however, in the presence of 90 to 120 mg. of ascorbic acid clotting did not occur and serotonin concentrations were substantially reduced. Good clotting, therefore, was deemed to be imperative for

reproducible results, and 41 mg. per 7 ml. of blood was chosen because numerous observations showed that satisfactory clotting consistently occurred with that amount. When 58 mg. was used a poorly formed clot occasionally was observed. Nevertheless, 58 mg. should be used for blood from patients with carcinoid syndrome since greater amounts of serotonin were found with that amount. The specimen is discarded if poor clot formation occurs.

In duplicate samples from normal individuals essentially constant concentrations of serotonin were noted, and the addition of increasing amounts of serotonin in an in vitro system did not result in an increased loss of serotonin, up to 5.0 μg per milliliter, with the use of 41 mg. of ascorbic acid per 7 ml. of blood. Excessive amounts of serotonin in plasma and adsorbed on platelets in the carcinoid syndrome appeared to be more readily destroyed, however, since the addition of 58 mg. of ascorbic acid apparently resulted in higher serotonin levels than when 41 mg. per 7 ml. of blood was used.

Because Weiner and Udenfriend[28] reported a "tendency" toward lower serotonin values in hospitalized patients with less than 10 Gm. of hemoglobin, the hematocrit was measured in 47 of the 50 normal individuals. The hematocrits varied from 32 (in a recent blood donor) to 55, but there was no correlation between the hematocrit and serotonin level, nor was any sex difference apparent in the 8 female donors (range 0.13 to 0.30 μg per milliliter). It has been stated that if all of the serotonin present in platelets was liberated in the serum the concentration would be 0.35 μg per milliliter.[8] The concentrations obtained in this study probably do not represent total release of serotonin into serum. Nevertheless, constant recovery is obtained by blocking destruction of serotonin, and serum values are found to be comparable to the previously reported values for normal blood.

SUMMARY

By blocking the destruction of serotonin with added ascorbic acid reproducible concentrations of serotonin in the serum are obtained, and a substantial recovery of serotonin added to whole blood can be demonstrated. The mean concentration of serum serotonin by this method in 50 normal persons was 0.21 μg per milliliter, with a range of 0.10 to 0.32 μg per milliliter (± 2 S.D.). Previously noted low and variable concentrations of serotonin in serum are believed to be due to substantial destruction of serotonin prior to assay.

I wish to thank Dr. C. J. Watson for his interest in this investigation and his advice in the preparation of this manuscript. The Farrand spectrofluorometer was used with the kind permission of Dr. Arnold Lazarow of the Department of Anatomy, University of Minnesota.

Addendum.—Since this paper was submitted for publication an additional method for determination of blood serotonin in man has appeared.[31]

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SERUM PHOSPHOLIPIDS IN CORONARY ARTERY DISEASE

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THE phospholipids in serum can be classified into 2 groups: those containing choline and those containing moieties other than choline (i.e., serine, ethanolamine, or inositol). The choline-containing phospholipids (CCPL) are (1) phosphatidyl choline (lecithin), (2) lysophosphatidyl choline, and (3) sphingomyelin. The noncholine-containing phospholipids (NCCPL), also known as the cephalins, are (1) phosphatidyl ethanolamine, (2) lysophosphatidyl ethanolamine, (3) phosphatidyl serine, and (4) phosphatidyl inositide(s).

Knowledge of the physiologic role of these serum phospholipids is scanty. They are known to be integral parts of serum lipoproteins[1] and in this form appear to be a necessary cofactor for the lipolytic activity of lipoprotein lipase.[2] Perhaps a more fascinating role is their as yet undefined function in normal coagulation and intravascular clotting. This role is suggested by the coagulant effect of cephalin compounds present in thromboplastin,[3, 3a] red blood cells,[4] platelets,[5] and serum[6] and by the in vitro and in vivo promotion or inhibition of coagulation by various phospholipids.[7, 8, 8a]

The association of intravascular clotting and lipid abnormalities with atherosclerosis, has suggested a possible association of lipid abnormalities and intravascular clotting. Because of the profound effect of phospholipids on experimental coagulation, we thought it would be of interest to investigate the distribution of the CCPL and NCCPL in the serum of a group of 20 patients with coronary artery disease. These patients in the main had probably suffered an episode of intravascular thrombosis as indicated by a previous myocardial infarction, or were potentially susceptible to such an event as indicated by the symptom of angina pectoris.

Our method of approach was to determine the total phospholipids and the choline- and noncholine-containing phospholipids in the serum of control subjects and patients with coronary artery disease. Our results indicate a rise in both the NCCPL and CCPL fractions neither of which by itself is statistically significant but together account for the statistically significant rise in phospholipids noted in patients with coronary artery disease.

METHODS

The techniques used have been described in detail in a previous publication.[9] Choline is assumed to be present in serum attached only to phospholipids. Trace amounts

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of acetylcholine[10] and unbound choline[11] are believed to be present, but for our present purposes may be considered negligible.

Choline was measured by microbiologic assay[9] using modified methods of Horowitz and Beadle[12] and Leucke and Pearson.[13] Lipid phosphorus was measured colorimetrically at 625 mμ after the method of Fiske and Subbarow.[14] This method consists of twice digesting a dried aliquot of an ethanol:ethyl ether (3:1) extract of serum with 10 N H_2SO_4 and Superoxol over a flame, and using 2,4-amino-naphthol-sulfonic acid for development of a blue color.

The following calculations were made:

Lipid phosphorus (lipid P) mg. per cent × 25 = phospholipid (PL) mg. per cent

$$\frac{\mu M \text{ choline}}{\mu M \text{ lipid P}} \times \text{PL mg. per cent} = \text{choline-containing phospholipids (CCPL) mg. per cent}$$

PL (mg. per cent) − CCPL (mg. per cent) = noncholine-containing phospholipids (NCCPL) mg. per cent

Total serum cholesterol determinations by the method of Bloor[15] were also performed.

Subjects.—Thirty-six male physicians and laboratory workers, 20 to 56 years of age, comprise the control group. This group was evaluated by physical examination, hemogram, urinalysis, fasting blood sugar, chest x-ray, basal metabolism test, and electrocardiogram. The normal subjects are divided into two age groups, 20 to 29 years, and 32 to 56 years. Each of these age groups is compared with the patient group which consists of 18 men and 2 postmenopausal women, 32 to 59 years of age, with coronary artery disease manifest by angina pectoris and/or previous myocardial infarction.

All lipid determinations were made on serum collected after an overnight fast. The data reported here represent 29 determinations made on 20 patients, and 60 determinations made on 36 control subjects. The average of replicate determinations on a subject was used in calculating the mean lipid values in each group. Blood was obtained from the patient group from one month to several years after the onset of clinical manifestations of their disease, but never during the first month following recovery from an acute myocardial infarction. Several patients were on Dicumarol or recently had this medication discontinued. Other medications included nitroglycerine, aminophylline, and barbiturates. None of these medications in the dosage used is known to influence the serum lipids studied.

RESULTS

Table I compares the lipid values of the coronary artery disease group and normal subjects, and lists the significance of differences of means as measured by Fisher's t test.[16] On the average, the CCPL represent 79.1 ± 1.14* per cent, 78.7 ± 0.855 per cent, and 77.9 ± 1.54 per cent of the serum phosphatides in the young normal, older normal, and patient groups, respectively. The NCCPL represent 20.9 ± 1.14, 21.3 ± 0.734, and 22.1 ± 1.57 per cent of the serum phosphatides in the young normal, older normal, and patient groups respectively. The mean serum phospholipids (mg. per cent) in patients with coronary artery disease is greater by 10.1 per cent than in the 2 normal groups and attains significance, a finding in agreement with previous reports.[17, 18] The per cent increases in NCCPL of 17.3 per cent and in CCPL of 8.2 per cent are not statistically significant by themselves, but collectively account for the significant rise in total phospholipids. The rises in serum cholesterol with age and the highly significant rise in coronary artery disease are as expected.

*Standard error.

TABLE I. THE DISTRIBUTION OF LIPIDS IN HEALTH AND CORONARY ARTERY DISEASE

SUBJECTS	AGE GROUPS	NUMBER OF SUBJECTS	PHOSPHOLIPIDS (MG. %)	CHOLINE CHLORIDE (MG. %)	CCPL		NCCPL		CHOLESTEROL MG. %
					MG. %	% PL	MG. %	% PL	
Normal	20-29	10	246 ± 3.83*	35.03 ± 1.72	194 ± 9.51	79.1 ± 1.14	51.9 ± 3.88	20.9 ± 1.14	246 ± 10.9
	32-56	26	247 ± 7.67	35.06 ± 1.19	195 ± 6.12	78.7 ± 0.855	52.4 ± 2.27	21.3 ± 0.734	277 ± 9.33
		P34†	N.S.†	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Coronary artery disease	32-59 vs. normals 20-29	20 P28	272 ± 8.26 <0.05	37.98 ± 1.14 N.S.	211 ± 6.17 N.S.	77.9 ± 1.54 N.S.	61 ± 4.90 N.S.	22.1 ± 1.57 N.S.	323 ± 12.6 <<0.01
	vs. normals 32-56	P44	<0.05	N.S.	N.S.	N.S.	N.S.	N.S.	<0.01

*Mean ± standard error.

†Probability that an observed difference of means is due to chance. The P refers to the degrees of freedom. N.S. means p >0.05.

Although not tabulated here, the fraction of the serum phosphatides represented by the CCPL and NCCPL was found to vary considerably. In control subjects, aged 20 to 56 years, the CCPL represents 71.5 to 90.4 per cent and the NCCPL 9.6 to 28.5 per cent of the serum phosphatides.[9] The corresponding ranges for CCPL and NCCPL in the patient group are 66.4 to 99.6 per cent and 0.4 to 33.6 per cent, respectively.

DISCUSSION

The data indicate that patients with coronary artery disease as a group have an increased serum phospholipid content as well as an increased serum cholesterol content. The increase in phospholipid appears to be accounted for by small rises in both the NCCPL and CCPL.

The data give no insight regarding which phospholipid species account for the rise in each fraction. The work of Kayden and Steele[19] is of interest as they reported that the sphingomyelin-cholesterol ratio in the two major lipoprotein fractions, as determined by starch electrophoresis, is constant even with increases in cholesterol that occur in coronary artery disease. The increase in CCPL noted here might be accounted for by an increased sphingomyelin content. There are no similar observations that bear on the distribution of the other phospholipid species in coronary artery disease.

The average values of CCPL and NCCPL in serum reported here are in substantial agreement with those of Albrink,[20] who reported 70 to 80 per cent of serum phospholipids are CCPL in normal subjects and numerous disease states, and in agreement with the reports in the older literature summarized by Witcoff.[21] However, the values reported here differ from the values found by Taurog and associates,[22] Hack,[23] and Sinclair[24] who believed less than 10 per cent of human plasma phospholipid is NCCPL. More recently, using silicic acid chromatography, Phillips[25] analyzed 7 normal sera and found less than 10 per cent of the serum phospholipid to be NCCPL.[25]

The data enumerated here establish no causal relationship between phospholipids and intravascular clotting or atherosclerosis. The data do emphasize such patients have an increased concentration of serum phospholipid, apparently of both the choline-containing and noncholine-containing types. These limited data do not warrant speculation regarding the role of these lipids in blood coagulation but when added to the findings reported in the excellent review by Poole[26] regarding the provocative role of certain phospholipids in accelerating in vitro coagulation is further suggestive evidence that the phospholipids may yet prove of great importance in resolving the imprecisely understood role of lipids in blood coagulation. These observations certainly suggest the need for further studies in the partition of the phospholipids in coronary artery disease as well as other disease states characterized in part or in the main by intravascular thrombosis.

SUMMARY

The mean concentration of serum phospholipids in a group of patients with coronary artery disease was found to be significantly greater than that of

controls. This increase seems to be accounted for by small increases in the mean concentration of both choline-containing and noncholine-containing phospholipids.

The helpful advice of Drs. Mary Lou Eilert and Richard Jeffrey Jones and the technical assistance of Oliver Rampersad and Sheldon Huffman are gratefully acknowledged.

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THE BASE BINDING PROPERTY OF THE SERUM PROTEINS WITH RESPECT TO MAGNESIUM

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MAGNESIUM in human serum exists in two forms, diffusible and nondiffusible. Copeland and Sunderman[1] have suggested that the magnesium proteinate in the serum acts as a dissociated salt according to the mass law relationship and showed that the diffusible magnesium is practically all ionic. These authors have shown that magnesium is bound to albumin.[1] Carr[2] has reported that the binding of magnesium in different protein solutions in vitro is similar to that of calcium. However, since the base binding property of serum albumin and different globulins with respect to magnesium in normal and diseased states has not been investigated, an attempt was made to study this aspect.

MATERIALS AND METHODS

In vitro binding in normal subjects was studied in samples of different protein fractions obtained by starch block electrophoresis.[3] Twenty-two fractions were obtained from each specimen of serum obtained from 5 normal subjects. Appropriate fractions were pooled into albumin and alpha-1, alpha-2, beta, and gamma globulin samples. To each of these different protein fractions was added from 5 to 8 ml. of magnesium chloride solution in normal saline with a concentration of approximately 2.0 mEq. of magnesium per liter, and the mixtures were incubated at 37° C. for 30 minutes. The volume of magnesium chloride solution added varied depending on the volume of the protein sample. Total and ultrafiltrable magnesium was then determined in each sample.[4] Ultrafiltration was done by a technique reported previously.[5] Protein concentration of each fraction was determined by the tyrosine method[6] after addition of magnesium chloride solution. From these, the amount of magnesium bound to different protein fractions was calculated. As a control, 10 ml. of the magnesium chloride solution in normal saline was subjected to ultrafiltration, and the magnesium was determined in both the original sample and its ultrafiltrate.

In vivo binding was studied on serum samples obtained from 4 groups: Group I, normal subjects; Group II, multiple myeloma; Group III, hyperglobulinemia (exclusive of multiple myeloma); and Group IV, hypoproteinemia. Cases with uremia were excluded. Serum total and ultrafiltrable magnesium were determined on each sample. Total protein was determined by the biuret method.[7] Paper electrophoresis was used to determine the different fractions of the serum proteins.[8] Nonultrafiltrable magnesium was calculated in millimoles per kilogram of water using the McLean and Hastings[9] formula for correction of the volume occupied by the solids and proteins in the sera. Different protein components were expressed as fractions of the total proteins. The albumin was expressed in

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TABLE I. MAGNESIUM-PROTEIN RELATIONSHIP IN VITRO IN NORMALS

	MAGNESIUM (mEq./L.)		PROTEIN	MAGNESIUM (mEq./L.)		PROTEIN	MAGNESIUM (mEq./L.)		PROTEIN	MAGNESIUM (mEq./L.)		PROTEIN	MAGNESIUM (mEq./L.)		PROTEIN
	TOTAL	UF	(MG. %)	TOTAL	UF	(MG. %)	TOTAL	UF	(MG. %)	TOTAL	UF	(MG. %)	TOTAL	UF	(MG. %)
Albumin (mEq. Mg bound/ Gm. albumin)	2.04	1.30		1.56	1.26	976	1.54	1.16	860	1.56	1.27	1,590	1.56	1.16	2,000
				0.03			0.04			0.018			0.02		
Alpha-1 globulin	1.72	1.52		1.70	1.68	90	1.68	1.52	205	1.86	1.67	350	1.78	1.73	250
Alpha-2 globulin	1.64	1.52		1.52	1.40	125	1.72	1.68	190	2.06	1.92	250	1.74	1.72	225
Beta globulin	1.04	0.96		1.66	1.63	280	1.28	1.23	250	1.96	1.84	350	1.66	1.65	370
Gamma globulin	1.06	1.24		1.42	1.27	580	0.78	0.80	405	0.98	1.08	600	1.40	1.45	400
Saline (control)				2.08	1.86					2.02	2.05		2.12	2.21	

UF = ultrafiltrate.

The pH was found to range from 7.9 to 8.0. Twenty-two fractions were obtained from each of 5 normal serum specimens. Appropriate fractions were pooled into albumin, alpha-1, alpha-2, beta, and gamma samples. $MgCl_2$ in normal saline solution was added to each sample. Protein and magnesium concentrations were then obtained, but, unfortunately, the protein concentrations were not obtained on the first series.

terms of millimoles per kilogram of water using the molecular weight of 69,000.[10] The nonultrafiltrable magnesium was plotted against the different fractions of the serum proteins on arithmetic graph paper. The molecular weight of albumin is well established and the nonultrafiltrable magnesium concentrations were plotted against the millimoles of albumin. In the instance of total proteins this calculation is not possible and in the instances of the other protein fractions would be nearly as difficult since each one is not actually homogeneous. Curves were determined by the method of least squares. The correlation coefficient and standard error for each group were calculated statistically,[11] and the P values were derived from Fisher's table (No. V.A.).[12]

RESULTS

Table I shows the results of in vitro experiments. Unfortunately, protein concentrations were not determined in the first series of protein fractions since the specimens were misplaced. The ultrafiltration results are included, nevertheless, since the results are qualitatively similar to the other 4 experiments. The magnesium which is not ultrafiltrable is probably bound to protein and will be described as being protein bound in this discussion. Roughly 0.027 mEq. of magnesium was bound to 1 Gm. of albumin. Besides albumin, alpha-1 and alpha-2 globulins also bound some magnesium in 3 out of 5 instances. The results obtained with beta globulin indicated equivocal binding in only one instance. In 4 out of 5 experiments magnesium in gamma globulin was completely ultrafiltrable indicating that this protein probably does not bind any magnesium. The concentration of the protein fractions, other than albumin, is small and makes binding property of respective fraction less certain. Magnesium chloride in saline solution was 90 to 104 per cent ultrafiltrable. These experiments also indicate that there is no selective adsorption of magnesium on the gauze or casing used for ultrafiltration.

Figs. 1 through 6 show in graphic form the relationship between the total nonultrafiltrable magnesium and the concentration of the various protein fractions. In the instances where significant correlations exist between nonultrafiltrable magnesium and a particular protein fraction, it can be inferred that the fraction has an affinity for magnesium. From these data it cannot be stated categorically that protein binding occurs. In the discussion which follows the relationship will be described by the term "probably bound."

Fig. 1 shows the curve for the total protein in grams per liter and the nonultrafiltrable magnesium in milliequivalents per liter in all the groups studied. The patients with increased total protein due to hypergammaglobulinemia were excluded since our in vitro results showed that gamma globulin does not bind any magnesium. A definite relationship between the total protein and the nonultrafiltrable magnesium was noted (correlation coefficient, r, = 0.75, $P < 0.01$). From this, roughly 1 Gm. of protein apparently binds 0.01 mEq. of magnesium. The pK magnesium proteinate in normal subjects was calculated to be 1.927 ± 0.052. From the curve, the relationship between total protein and the nonultrafiltrable magnesium can be expressed as $y = mx + b$, where y represents the nonultrafiltrable magnesium, x the total protein, m the slope of the curve, and b the intercept. Assuming that the constant b represents the nonultrafiltrable magnesium which is independent of the protein in

the sera, roughly 0.14 mEq. of nonultrafiltrable magnesium per liter must be accounted for by some substances other than the proteins.

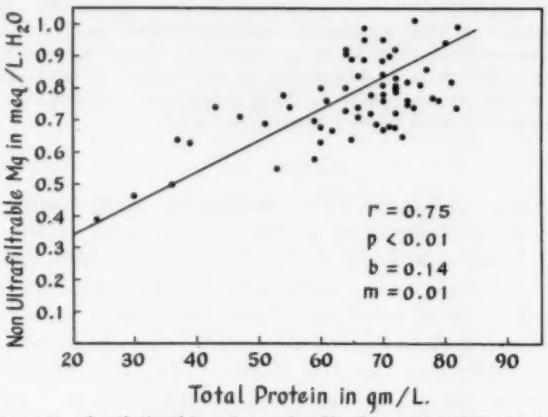

Fig. 1.—The curve of relationship of nonultrafiltrable magnesium to total serum proteins is plotted by the method of least squares. There is a significant positive correlation.

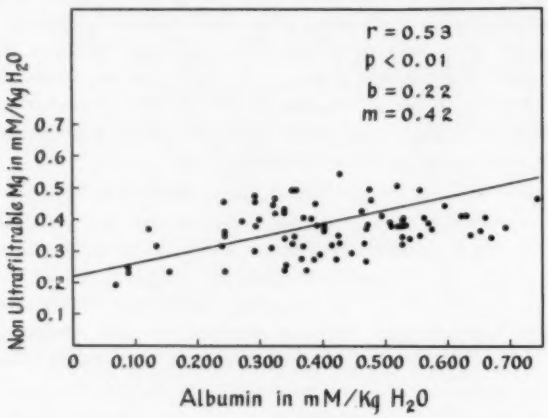

Fig. 2.—The relationship of albumin to nonultrafiltrable magnesium is plotted. There is significant positive correlation.

Fig. 2 shows the curve for the albumin and the nonultrafiltrable magnesium relationship for all the 4 groups when both were expressed in millimoles per kilogram of water. A significant correlation between the two was observed ($r = 0.5$, $P < 0.01$). From our calculations, approximately 0.012 mEq. of magnesium appeared to be bound per gram of albumin. From 60 to 65 per cent of the nonultrafiltrable magnesium could be accounted for by the albumin.

Fig. 3 shows the curve for alpha-1 globulin and nonultrafiltrable magnesium relationship: Group I, $r = 0.75$, $P < 0.01$; Group II, $r = 0.79$, $P < 0.02$. No correlation in Groups III and IV was observed.

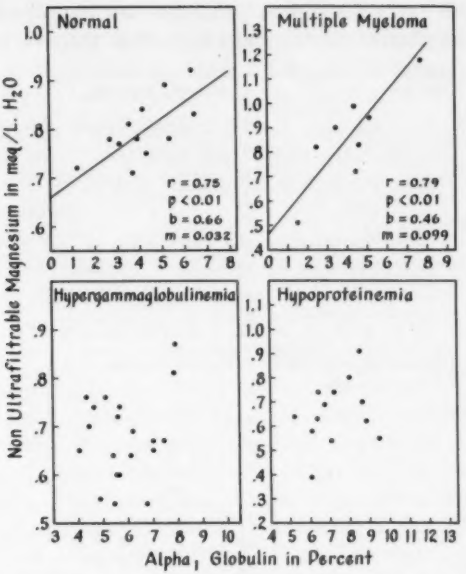

Fig. 3.—The relationship of alpha-1 globulin and nonultrafiltrable magnesium is plotted separately for each group. A positive correlation occurs only in normal subjects and patients with multiple myeloma.

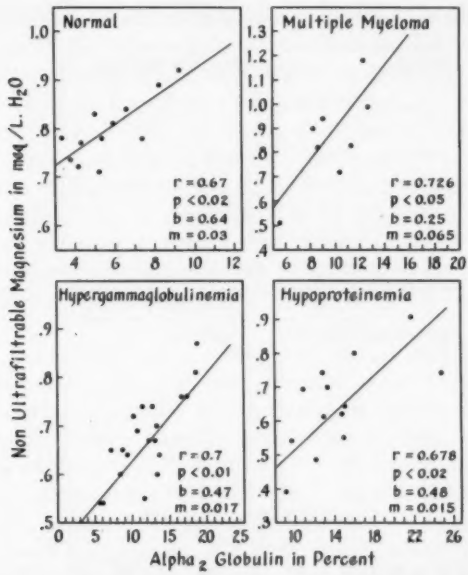

Fig. 4.—The relationship of alpha-2 globulin and nonultrafiltrable magnesium is plotted separately for each group. Positive correlation exists in all groups.

Fig. 4 shows the curve for alpha-2 globulin and nonultrafiltrable magnesium relationship. A significant correlation between the two was found in all

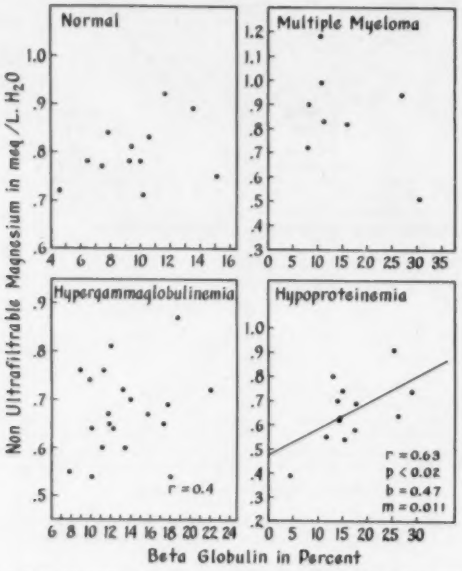

Fig. 5.—The relationship of beta globulin and nonultrafiltrable magnesium is plotted separately for each group. Only in the instance of hypoproteinemias does a positive correlation exist.

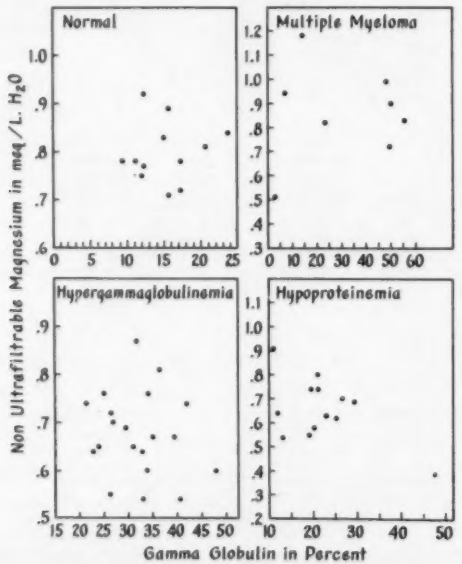

Fig. 6.—The relationship of gamma globulin and nonultrafiltrable magnesium is plotted for each group. Note absence of any correlation.

the groups (Group I, $r = 0.67$, $P < 0.02$; Group II, $r = 0.726$, $P < 0.05$; Group III, $r = 0.7$, $P < 0.01$; and Group IV, $r = 0.678$, $P < 0.02$). This indicates that alpha-2 globulin probably binds some magnesium in all the groups studied.

Fig. 5 shows the relationship between beta globulin and nonultrafiltrable magnesium. No correlation was observed in Groups I, II, and III. In Group IV, $r = 0.63$ and $P < 0.05$ indicating that some magnesium was probably bound to beta globulin.

Fig. 6 shows the scattergram for gamma globulin and nonultrafiltrable magnesium. No correlation between the two was found to exist in any group. Gamma globulin, therefore, does not seem to bind any magnesium in the groups studied.

DISCUSSION

From in vitro and in vivo studies, it appears that albumin binds magnesium whereas beta and gamma globulins do not. Besides albumin, alpha-2 globulin was another fraction which appeared to bind magnesium consistently in all the groups studied. Alpha-1 globulin in multiple myeloma and normal persons and beta globulin in hypoproteinemia also appeared to bind magnesium. pK magnesium proteinate as calculated from our data was 1.927 ± 0.052 which is very close to that reported by Copeland and Sunderman[1] (1.77 ± 0.15). In normal subjects, calcium appears to be bound to albumin and beta globulin,[13] whereas magnesium is bound to albumin and alpha-2 globulin.

From our analysis roughly 0.012 mEq. of magnesium was bound to 1.0 Gm. of albumin in the groups of patients studied. Copeland and Sunderman[1] reported that 0.013 ($\pm$ 0.004) mEq. of magnesium was bound to 1.0 Gm. of albumin. However, from our in vitro results in 4 normal subjects when albumin was obtained by means of starch block electrophoresis, the mean value for magnesium apparently bound to 1.0 Gm. of albumin was 0.027 mEq. The pH was above the physiologic range, however, in the in vitro experiments.

The analysis of our results also indicate that a small fraction of nonultrafiltrable magnesium may be accounted for by substances other than the proteins. However, there is no evidence at present as to which other substances in the sera could be responsible for binding magnesium besides the proteins. Drinker and Zinsser[14] have reported that besides protein, calcium is also bound to cephalin.

The fact that there is a positive correlation between concentration of nonultrafiltrable magnesium and concentration of total proteins and of several fractions makes the probability strong that these proteins "bind" magnesium. The in vitro studies with separated fractions strengthen this contention. There is no evidence whatever as to the kind of bond or bonds. However, the magnesium-protein relationship obeys the law of mass action, and the magnesium bound to protein is in a reversible combination. As in the case of calcium, it is probably correct to presume that there are as many undissociated magnesium proteinates as there are discrete proteins in plasma and therefore the nonultrafiltrable magnesium moiety should be regarded as the summation of a large number of different protein-magnesium complexes.

SUMMARY

1. The pK magnesium proteinate in normal subjects was found to be 1.927 ± 0.052.

2. Roughly 10 to 15 per cent of the nonultrafiltrable magnesium (in the normal subjects), appears to be independent of the serum proteins.

3. From statistical analysis roughly 1 Gm. of albumin bound to 0.012 mEq. of magnesium. Approximately 60 to 65 per cent of the nonultrafiltrable magnesium could be accounted for by the serum albumin in normal persons.

4. Alpha-1 globulin appeared to bind some magnesium in multiple myeloma and normal persons, whereas alpha-2 globulin apparently bound magnesium in all the groups studied.

5. Beta globulin appeared to bind some magnesium in patients with hypoproteinemia. In other groups it did not bind any magnesium.

6. Gamma globulin does not bind any magnesium, either in vivo or in vitro.

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THE EFFECT OF EXPERIMENTAL HEPATIC NECROSIS ON THE VITAMIN B_{12} CONTENT IN SERUM AND LIVER

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ALTHOUGH a significant increase of serum vitamin B_{12} levels has been reported[1-4] in patients with some of the liver diseases, the underlying mechanism for this increase has not been clarified, and no observations are available as to the correlation between the histopathologic change of the liver and the change of serum vitamin B_{12}. The increase of the vitamin B_{12} levels in patients with acute hepatitis, especially those with hepatic coma, was greater than in patients with cirrhosis of the liver, and there was also a greater proportional rise in the free form of serum vitamin B_{12}. On the other hand, the serum of patients with cirrhosis of the liver showed a greater increase in the bound form.[4] In patients with acute hepatitis, our clinical data have shown that (1) the increase of serum vitamin B_{12} levels occur during the acute phase of the disease; (2) the levels decline gradually after the disappearance of manifest symptoms of the disease; and (3) the increase seems to be related to acute hepatic damages rather than to the regeneration of the hepatic tissues.

Therefore, to elucidate the mechanism of this increase, experimental acute hepatic necrosis was produced in rabbits by a single injection of carbon tetrachloride (CCl_4). Serial determinations were made of the vitamin B_{12} levels in the serum and liver of intoxicated rabbits, and the histochemical findings were noted. The time and quantitative relationships between changes in the serum vitamin B_{12} levels and the liver vitamin B_{12} levels were studied. A preliminary report of certain phases of the study has been published already.[5]

MATERIALS AND METHODS

Acute hepatic necrosis was produced in rabbits by the subcutaneous injection of 0.5 or 1.0 ml. per kilogram of body weight of a 20 per cent solution of CCl_4 in olive oil. The animals had been kept ad libitum on a natural diet in our laboratory for at least a week before being used in the experiments. The animals weighed from 2.5 to 3.0 Kg. The total number of rabbits used was 29. In order to make serial observations with the same animal on the changes of vitamin B_{12} levels in the serum and liver, samples of blood and liver were obtained without killing the animals. Blood was withdrawn by heart puncture, and tissue blocks, 200 to 300 mg., of the liver were resected from the left lobe of the liver by aseptic operation under light ether anesthesia. For the determinations of weight and vitamin B_{12} content of the whole liver, the animals were sacrificed by cutting the carotid artery.

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The vitamin B_{12} content was determined with *Lactobacillus leichmannii* (A.T.C.C. 4797) by an acidimetric adaptation of the U.S.P. method with some modification.[6] As has been observed in human serum,[6-10] the vitamin B_{12} activity of rabbit serum obtained after heating for 30 minutes at 100° C. is much higher than that obtained without heating. Rabbit serum has levels of total vitamin B_{12} about a hundred times higher than human serum. For the assay of the total vitamin B_{12}, rabbit serum was diluted to 1:1,000 with pH 4.6 acetate buffer in a final concentration of 0.01 M and heated for 30 minutes at 100° C. After cooling and centrifugation, the supernatant was adjusted to pH 6.8 with K_2HPO_4 solution and used for the assay. The "free" or "easily available" form of serum vitamin B_{12} was assayed after the serum was diluted to 1:500 with sterilized distilled water, heated for 30 minutes at 56° C., and then added aseptically to the assay medium. The bound form of serum vitamin B_{12} was determined subtracting the value obtained for the "free" form from the total vitamin B_{12} levels.

Liver vitamin B_{12} was extracted as described by Dawbarn and Hine[11] by heating 1 per cent liver homogenates for 30 minutes at 100° C. in 0.1 M acetate buffer (pH 4.6) containing 5 mg. of potassium cyanide per 100 ml. After cooling and centrifugation, the supernatant obtained was diluted appropriately, adjusted to pH 6.8, and used for the assay.

The resected tissue blocks of the liver were also examined histologically. The tissues were stained as follows: (1) by hematoxylin and eosin, (2) for fat by Sudan III, (3) for glycogen by the method of Hotchkiss,[12] (4) for ribonucleic acid with methyl green-pyronine stain and ribonuclease digestion,[13] and (5) for desoxyribonucleic acid by the Feulgen reaction.[14]

To prepare the nuclear, mitochondrial, microsomal, and supernatant fluid fractions of rabbit liver, differential centrifugation was used as described by Schneider and Hogeboom.[15] The liver tissues were homogenized in 0.25 M sucrose solution. The nuclear and mitochondrial fractions were washed twice and the microsomal fraction was washed once with the sucrose solution. The method for extraction of vitamin B_{12} from each fraction was similar to that used for the whole homogenate. Nitrogen content in each fraction was determined by the micro-Kjeldahl method.

The paper electrophoretic method of Kunkel and Tiselius[16] as modified by Kobayashi[17] was used to show the electrophoretic behavior of vitamin B_{12} in serum and liver homogenates. Paper electrophoresis was carried out in triplicate with 0.01 to 0.02 ml. of serum or liver homogenate on TOYO No. 50 paper strips, 3 by 35 cm., with barbital buffer of 0.1 ionic strength, pH 8.6, for a period of 8 to 10 hours at 0.5 Ma. per centimeter width of paper. In the case of liver homogenates, an equal quantity of rabbit serum was applied to a fourth strip as a control for protein migration. The strips were air dried; one of these and a control strip were stained with bromphenol blue to locate the protein band. The other strips were cut into each fraction, or 1 cm. wide pieces, and each piece was placed in a test tube to which had been added 2.5 ml. of 0.01 M phosphate buffer, pH 6.8, and the tubes were heated for 30 minutes in a boiling water bath. After cooling, equal quantities of double strength medium were added to all tubes, and the assay of vitamin B_{12} was completed.

RESULTS

Histologic Changes.—Histologic changes in a section stained with hematoxylin and eosin are as follows. At about 24 hours after administration with 1 ml./Kg. B.W. of 20 per cent CCl_4 solution, liver cells in the central region of the liver lobules underwent necrosis followed by karyorrhexis and karyolysis. The adjacent cells in the middle region were swollen. At about 48 hours the situation was almost the same as at 24 hours except that the necrotic areas were slightly extended. On the fourth day necrosis was confined to the area around the sublobular veins. The intermediate area between this necrotic

area and the viable peripheral cells was comprised of a number of large cells, most of which were the dividing regenerating cells. On the seventh day necrotic cells were removed and replaced by large, newly regenerated cells. The hepatic cells in the central and middle regions were still slightly large, and the lining of these cells was irregular. On the twelfth day the histologic view of the liver tissues had become almost completely normal.

TABLE I. CHANGES IN LIVER FAT OF RABBITS GIVEN CCl_4 (SUDAN III STAIN)

DAYS AFTER CCl_4 ADMINISTRATION	CENTRAL ZONES	MID-ZONES	PERIPHERAL ZONES
Before	±	±	±
1	+++	++	±
2	+++	++	±
4	+	+	±
7	±	±	±
12	±	±	±

TABLE II. CHANGES IN LIVER GLYCOGEN OF RABBITS GIVEN CCl_4 (PAS STAINING AFTER THE HOTCHKISS METHOD)

DAYS AFTER CCl_4 ADMINISTRATION	CENTRAL ZONES	MID-ZONES	PERIPHERAL ZONES
Before	+++	+++	±
1	-	-	+
2	-	-	+
4	±	±	++
7	+	+	+
12	+++	+++	±

TABLE III. CHANGES IN LIVER RNA OF RABBITS GIVEN CCl_4 (METHYL GREEN-PYRONINE STAIN)

DAYS AFTER CCl_4 ADMINISTRATION	CENTRAL ZONES	MID-ZONES	PERIPHERAL ZONES
Before	++	++	++
1	-	±	±
2	-	+	+
4	+	+++	++
7	+++	+++	++
12	++	++	++

TABLE IV. CHANGES IN LIVER DNA OF RABBITS GIVEN CCl_4 (DNA STAINING BY FEULGEN REACTION)

DAYS AFTER CCl_4 ADMINISTRATION	CENTRAL ZONES	MID-ZONES	PERIPHERAL ZONES
Before	+	+	+
1	±	+	+
2	±	+	+
4	+	++	++
7	++	++	++
12	+	+	+

Histochemical changes in the liver tissues are summarized in Tables I to IV. As will be seen, the most striking changes were the deposition of fat and the disappearance of glycogen and pyroninophilic substance in the central and middle regions at about 24 to 48 hours after CCl_4 administration. Thereafter these changes tended to recover rapidly and, on the seventh or twelfth

day, the histochemical view of the liver tissue was almost the same as the normal liver. The temporary increase of glycogen in the peripheral cells after CCl_4 administration was characteristic of the histochemical changes of glycogen, and this was in good contrast with usual scantiness of glycogen in normal liver. Later, however, the glycogen content in the peripheral cells again was decreased. The development and the recovery of the histochemical changes corresponded with those of the central lobular necrosis observed in sections stained with hematoxylin and eosin.

TABLE V. SERUM VITAMIN B_{12} CONCENTRATIONS OF RABBITS BEFORE AND AFTER CCl_4 ADMINISTRATION

NO. OF RABBITS	SERUM B_{12} CONC. BEFORE CCl_4 ADMINISTRATION (mμg/ML.)		20 PER CENT CCl_4 DOSE INJECTED (ML./KG. B. W.)	SERUM B_{12} CONC. AT 24 HR. AFTER CCl_4 ADMINISTRATION (mμg/ML.)		SERUM B_{12} CONC. AT 48 HR. AFTER CCl_4 ADMINISTRATION (mμg/ML.)	
	FREE FORM	TOTAL		FREE FORM	TOTAL	FREE FORM	TOTAL
1	19.0	67.0	1.0	19.0 (100)*	141.0 (210)*	44.0 (231)*	244.0 (349)*
2	10.5	49.0	1.0	14.0 (133)	64.0 (130)	4.5 (43)	136.0 (278)
3	14.4	36.0	1.0	—	—	77.6 (539)	135.5 (376)
4	29.6	72.0	1.0	—	—	57.6 (194)	262.0 (364)
5	26.3	49.0	1.0	—	—	35.5 (135)	200.0 (408)
6	21.8	44.0	0.5	—	—	43.8 (201)	102.0 (234)
7	12.5	50.0	0.5	—	—	15.0 (120)	148.0 (296)
8	15.0	50.5	0.5	—	—	20.5 (137)	129.5 (256)
9	14.8	43.0	—	—	—	14.6 (92)	36.0 (84)
10	9.8	16.0	—	6.0 (61)	15.0 (94)	8.0 (81)	16.0 (100)
Average	17.4	47.7					

*$\frac{B_{12} \text{ conc. after } CCl_4 \text{ administration}}{B_{12} \text{ conc. before } CCl_4 \text{ administration}} \times 100$ (per cent).

In the rabbits given only 0.5 ml./Kg. B.W. of 20 per cent CCl_4 solution, the necrotic area in the liver lobules was slightly smaller than in those given 1 ml. Nevertheless, the characteristics and the course of the histologic changes were similar in both cases.

In summary, the characteristics of liver damage after CCl_4 administration were the following: central necrosis of the liver lobules, followed by the deposition of fat and the disappearance of glycogen and the pyroninophilic substance in central and middle regions of the lobules. These data have been already cited by many workers.[19, 20]

Effect of CCl_4 Administration on Serum Vitamin B_{12} Levels of Rabbits.— In this experiment 10 rabbits were divided into 3 groups as shown in Table V. Five were given 1.0 ml. and three 0.5 ml. of 20 per cent CCl_4 solution, the third group served as a control and received nothing. Blood was obtained by heart puncture before and at 24 and 48 hours after CCl_4 administration. The serum specimens were assayed for the vitamin B_{12} activity, and the results in Table V show that the serum vitamin B_{12} levels were increased remarkably after CCl_4 administration. In the animals given 1.0 ml. of 20 per cent CCl_4 per kilogram of body weight, the average increase of total vitamin B_{12} activity in the serum at 48 hours after the administration of CCl_4 was 3.6 times normal. In those given 0.5 ml. of 20 per cent CCl_4, the increase was 2.6 times

normal. The difference in these values almost corresponds to the difference in extent of the necrotic areas in the liver tissues of both cases. The free form of serum vitamin B_{12} was also increased about 1.3 to 5.4 times at 48 hours after CCl_4 administration except for Rabbit 2, and, in this case, an increase was noted later.

To ascertain whether the increase of the vitamin B_{12} activity in the serum was due to true vitamin B_{12}, the changes in vitamin B_{12} activity of serum supernatant (obtained as described above) by heating in NaOH solution at a final concentration of 0.1 N for 30 minutes at 100° C. were examined.[9] The results are shown in Table VI. Alkali stable fraction of vitamin B_{12} activity was only about 5 per cent of the total activity in both sera before and after CCl_4 administration. It is evident that the increase of the serum vitamin B_{12} activity after CCl_4 administration is due to vitamin B_{12} itself almost entirely.

TABLE VI. EFFECT OF ALKALINE HEATING ON SERUM VITAMIN B_{12} OF RABBITS BEFORE AND AFTER CCl_4 ADMINISTRATION

NO. OF RABBITS	CCl_4 ADMINISTRATION	SERUM B_{12} CONC. (mμg/ML.)	
		TOTAL	B_{12} ACTIVITY AFTER ALKALINE HEATING
3	Before	36.0	2.4 (6.6)*
3	After	135.5	7.6 (5.7)
4	Before	72.0	3.6 (5.0)
4	After	262.0	12.8 (4.9)

*Ratio to total vitamin B_{12} concentration (per cent).

Fig. 1 shows the course of the serum vitamin B_{12} levels of Rabbits 1 and 2 given 1 ml. of 20 per cent CCl_4 with Rabbit 10 serving as a control. The total serum vitamin B_{12} levels reached their maximum within 2 or 4 days and continued for about 10 days; thereafter they declined gradually and returned to pretreatment values by the thirty-first day. The free form of the serum vitamin B_{12} also reached its maximum within 2 or 4 days and then returned to the pretreatment values by the end of the first week.

Fig. 2 shows the course of the serum vitamin B_{12} levels of Rabbits 6 and 7 which were given 0.5 ml. of 20 per cent CCl_4. The total serum vitamin B_{12} levels reached their maximum within 2 or 4 days, as did those of the rabbits shown in Fig. 1, but the maximum levels were lower and the duration shorter. The total vitamin B_{12} levels had returned to the pretreatment values by 10 or 18 days. The free form of the serum vitamin B_{12} was increased and declined in the same manner as in Rabbits 1 and 2 (Fig. 1).

In the illustrations a close time relationship will be noticed between the development of the acute hepatic necrosis and the elevation in total serum vitamin B_{12} levels after CCl_4 administration. The free form of serum vitamin B_{12} was increased temporarily and declined after the second or fourth day when the hepatocellular disintegration ceased and the removal of the debris was in progress. The total vitamin B_{12} levels in serum had begun to decline when the regeneration of the liver tissue was almost completely finished. In

the rabbits given 1.0 ml. of 20 per cent CCl_4, serum vitamin B_{12} levels returned to pretreatment values in about 1 month, while the time in those animals given 0.5 ml. was 10 to 20 days.

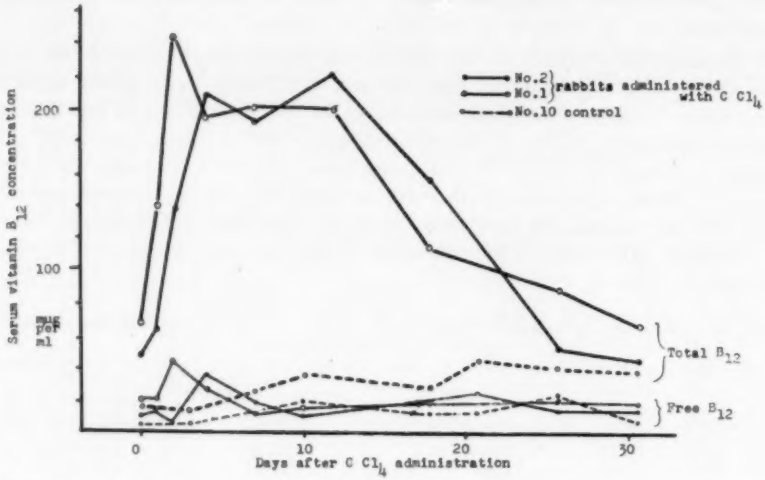

Fig. 1.—Changes in the serum vitamin B_{12} concentration of rabbits given 1.0 ml. of 20 per cent CCl_4 per kilogram of body weight.

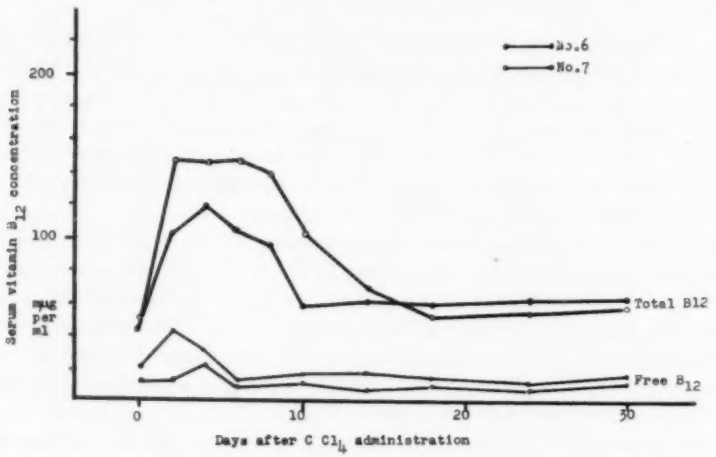

Fig. 2.—Changes in the serum vitamin B_{12} concentration of rabbits given 0.5 ml. of CCl_4.

Distribution of the Vitamin B_{12} Activity in Paper Electrophoretic Fractions of Rabbit Serum Before and at 48 Hours After CCl_4 Administration.—Typical results showing the distribution of the vitamin B_{12} in the electrophoretic fractions of serum are given in Fig. 3. It will be seen that the vitamin B_{12} activity of normal rabbit serum was found mainly in alpha and beta

globulin fractions. The peak of the greatest vitamin B_{12} activity corresponded with the alpha globulin band, and increases in vitamin B_{12} after CCl_4 administration were found in the same fractions. Electrophoretic patterns of rabbit serum protein were less definite than those of human serum protein. From the electrophoretic study the increase in serum vitamin B_{12} after CCl_4 administration could not be differentiated from the vitamin B_{12} naturally present in the serum.

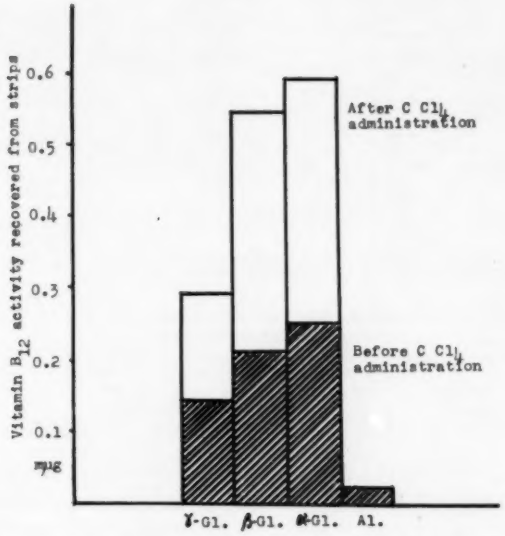

Fig. 3.—Distribution of the serum vitamin B_{12} in a rabbit before and after CCl_4 administration (0.1 ml. of serum from Rabbit 7 before and at 48 hours after injection was used).

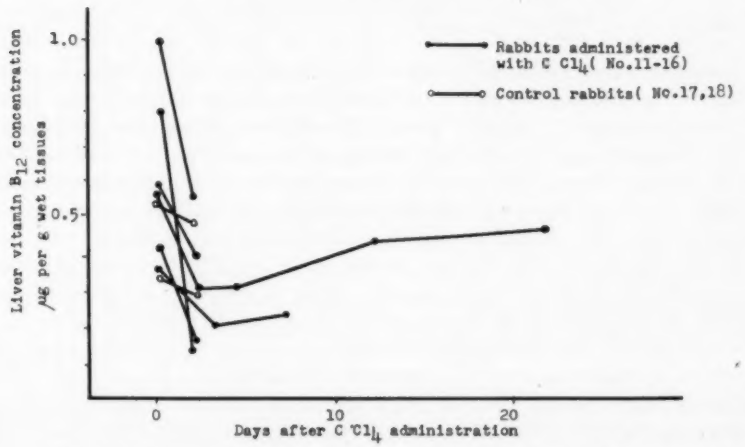

Fig. 4.—Changes in the liver vitamin B_{12} concentration of rabbits given CCl_4.

Effect of CCl_4 Administration on the Vitamin B_{12} Content of Rabbit Livers.—The vitamin B_{12} content of the liver tissues of untreated rabbits is shown in Table VII. The liver vitamin B_{12} levels of untreated normal rabbits ranged from 0.34 to 1.00 μg per Gm. of wet tissues with a mean of 0.58

TABLE VII. VITAMIN B_{12} CONCENTRATIONS OF RABBIT LIVERS BEFORE AND AFTER CCl_4 ADMINISTRATION

NO. OF RABBITS	CCl_4 DOSE INJECTED (ML./KG. B. W.)	LIVER B_{12} CONC. BEFORE CCl_4 ADMINISTRATION (μg PER GM. WET TISSUE)		INTERVAL AFTER CCl_4 ADMINISTRATION (HOURS)	LIVER B_{12} CONC. AFTER CCl_4 ADMINISTRATION (μg PER GM. WET TISSUE)	
11	1.0	0.59	Average of Rabbits 11 to 16 0.62	48	0.49 (83)*	Average of Rabbits 11 to 16 0.30 (50)
12	1.0	0.80		48	0.12 (15)	
13	1.0	0.57		48	0.30 (53)	
14	1.0	1.00		48	0.56 (56)	
15	1.0	0.42		48	0.15 (36)	
16	1.0	0.36		72	0.20 (55)	
17	—	0.57		—	0.48 (84)	
18	—	0.34		—	0.30 (88)	

*Ratio to B_{12} concentration before CCl_4 administration (per cent).

TABLE VIII. CHANGES IN VITAMIN B_{12} CONTENT AND WEIGHT OF WHOLE LIVER OF RABBITS AFTER CCl_4 ADMINISTRATION

TREATMENT	NO. OF RABBITS	WEIGHT OF LIVER (GM.)	LIVER B_{12} CONC. (μg PER GM. WET TISSUE)	B_{12} CONTENT OF WHOLE LIVER (μg)
CCl_4-intoxicated rabbits	21	98.0	0.26	25.48
	22	92.5	0.24	22.20
	23	95.0	0.36	34.20
	24	88.0	0.40	35.20
	Average	93.4	0.32	29.27
Nontreated rabbits	26	75.5	0.54	40.77
	27	86.0	0.47	40.42
	28	72.0	0.71	51.12
	29	64.0	0.96	61.44
	Average	74.4	0.67	48.44

μg. To determine the change in the liver vitamin B_{12} levels of the same animal after CCl_4 administration, a small block (as much as 200 to 300 mg. of wet liver tissue) was resected at laparotomy. Twenty-four hours later 1.0 ml. of 20 per cent CCl_4 was injected, and, at various time intervals, liver tissue was resected in the same way. As a control to examine the effect of the resection of a small block of liver tissue, 2 animals were similarly treated without the injection of CCl_4. The results are shown in Table VII and in Fig. 4. The vitamin B_{12} content of the liver tissues of rabbits given CCl_4 had been decreased markedly within 48 hours. The extent of the decrease of the liver vitamin B_{12} levels of 6 rabbits at 48 hours after CCl_4 administration was from 17 to 85 per cent of the pretreatment values with a mean of 50 per cent, while that of 2 control rabbits was 13 and 15 per cent with a mean of 14 per cent. From these mean values, 36 per cent of the decrease of the liver vitamin B_{12} levels might be attributed to CCl_4 intoxication. The decrease of the liver vitamin B_{12} levels began to recover from the effect of CCl_4 injection after the fourth day. In

a case which was examined for 22 days after CCl_4 injection, the extent of recovery of the liver vitamin B_{12} levels was 80 per cent of the pretreatment value. In this respect the recovery of the vitamin B_{12} storage in the liver seemed to be slower than that of the histologic damage. However, because the slight decrease of the vitamin B_{12} content of the liver tissues was observed even after the resection of a small block of the liver tissue, the influence of the repeated partial hepatectomies on the retardation of the recovery of the vitamin B_{12} storage in the liver had to be considered.

TABLE IX. INTRACELLULAR DISTRIBUTION OF VITAMIN B_{12} IN RABBIT LIVER HOMOGENATES

	NORMAL LIVER					LIVER OF A RABBIT GIVEN CCl_4*				
	CONTENT OF B_{12}		CONTENT OF NITROGEN		CONTENT OF B_{12}	CONTENT OF B_{12}		CONTENT OF NITROGEN		CONTENT OF B_{12}
FRACTIONS	mμg PER GM. WET TISSUE	PER CENT	MG. PER GM. WET TISSUE	PER CENT	(mμg/MG. N)	mμg PER GM. WET TISSUE	PER CENT	MG. PER GM. WET TISSUE	PER CENT	(mμg/MG. N)
Whole homogenates	540		38.5		14.0	260		31.4		8.3
Nuclear fraction	57	10.8	7.8	21.0	7.3	48	19.0	7.4	24.2	6.5
Mitochondrial fraction	281	53.3	6.9	18.6	40.7	128	50.8	5.8	19.0	22.1
Microsomal fraction	8	1.5	6.0	16.2	1.3	5	1.9	5.2	17.0	1.0
Supernatant fluid fraction	182	34.4	16.4	44.2	11.1	71	28.2	12.2	39.9	5.8
Total of fractions	528	100	37.1	100		252	100	36.6	100	
Recovery (per cent)	97.7		96.3			97.4		97.4		

*Dose of CCl_4 administered: 1.0 ml. per Kg. body weight of 20 per cent CCl_4.

To determine the changes in vitamin B_{12} content of the whole liver after CCl_4 administration, 4 untreated rabbits and 4 rabbits given 1.0 ml. of 20 per cent CCl_4 48 hours previously were sacrificed. The vitamin B_{12} content and total weight of the livers were estimated, and the results are shown in Table VIII. It will be seen that the total weight of the liver was slightly higher and vitamin B_{12} content was markedly lower after CCl_4 administration. When compared to average values, the vitamin B_{12} content of the whole liver of rabbits given CCl_4 was decreased to almost half that found in the livers of untreated rabbits. From the data shown in Table V it can be estimated that the average value of the vitamin B_{12} amount which was increased in the serum within 48 hours after 1.0 ml. CCl_4 was 141 mμg per milliliter. If the amount of the total serum circulating in a whole body is 4.5 per cent of body weight,[18] the average amount of the total serum of rabbits weighing 2.5 Kg. is 113 ml. Then the vitamin B_{12} amount which was increased in the total serum circulating in a whole body within 48 hours after CCl_4 administration was 15.9 μg. It may be compatible with the value (19.2 μg) which was the difference between the average vitamin B_{12} contents of the whole livers of untreated rabbits and CCl_4-intoxicated rabbits.

Intracellular Distribution of the Liver Vitamin B_{12} in Both Normal and CCl_4-Intoxicated Rabbits.—Liver tissue homogenates from both a normal rabbit and a rabbit given 1.0 ml. of 20 per cent CCl_4 48 hours previously were

fractionated by centrifugation into nuclear, mitochondrial, microsomal, and supernatant fluid fractions. The vitamin B_{12} content of whole homogenates and also of each fraction was determined by the same procedures as described before. The results are shown in Table IX. Vitamin B_{12} in the normal liver cells was distributed mainly in mitochondrial and supernatant fluid fractions and scarcely in other fractions. Although some of the vitamin B_{12} activity in the nuclear fraction was shown, it might have been due partially to cell debris retained in this fraction. The vitamin B_{12} content in mitochondrial fraction was the highest, above half that found in whole cells. In the fatty liver due to experimental hepatic necrosis the vitamin B_{12} contents in whole homogenates and in mitochondrial and supernatant fluid fractions were decreased significantly. The degree of the decrease was slightly higher in the supernatant fluid than in the mitochondrial fraction. In nuclear and microsomal fractions the decrease of vitamin B_{12} was not as significant as in the microchondrial and supernatant fluid fractions.

Distribution of Vitamin B_{12} Activity in Paper Electrophoretic Fractions of Rabbit Liver Homogenates.—Paper electrophoresis of liver homogenates was used to clarify the electrophoretic mobility of vitamin B_{12} in rabbit liver cells. Ten per cent homogenates of the liver tissue from a normal rabbit in 0.25 M sucrose solution was prepared. Two examinations were made: one was the suspension after removal of cell debris and nuclear fraction and the other was the supernatant fluid after removal of all particulate fractions. The results are shown in Figs. 5 and 6. In the liver tissue suspensions which included particulate fractions there were two peaks of greatest vitamin B_{12} activity. One corresponded to the point of application of the material to the paper and the other to the beta globulin band on the control serum strip. In the case of the supernatant fluid fraction, however, there was only one peak of greatest vitamin B_{12} activity and it corresponded to the serum beta globulin band. From these results it is apparent that the vitamin B_{12} activity deposited at the point of application is due to particulate materials, especially to mitochondrias. As shown in Fig. 5, the vitamin B_{12} activity remaining near the origin of application of the homogenates is about 60 per cent of the total vitamin activity recovered from the paper strip. If the particulate materials in the homogenates without the nuclear fraction were deposited at the point of application, this value may be compatible with the value calculated from the results shown in Table IX.

Effect of CCl_4 Administration on Urinary Excretion of Vitamin B_{12} in Rabbits.—To determine changes in the urinary excretion of vitamin B_{12} during CCl_4 intoxication, the vitamin B_{12} content in every 24-hour urine collection was measured in Rabbits 1 and 2 for a period of 33 days (3 days before to 30 days after CCl_4 administration). Vitamin B_{12} in the urine appeared to be in a free form and no additional vitamin B_{12} activity was obtained by heating the urine. Results are shown in Figs. 7 and 8. It is seen that the 24-hour urine quantities were diminished markedly for a week after CCl_4 administration, probably due to a toxic nephrosis provoked by CCl_4, with a

concomitant decrease in the vitamin B_{12} content. Urine quantities and the vitamin B_{12} excretion then returned to normal until 10 days after CCl_4 administration at which time there was an increase. The vitamin B_{12} excretion, in particular, was markedly elevated until 22 days after CCl_4 administration. The elevation then declined gradually, and the values were almost normal after a total of 30 days had passed. The time relationship between changes of the urinary vitamin B_{12} excretion and the serum vitamin B_{12} levels can be clearly detected.

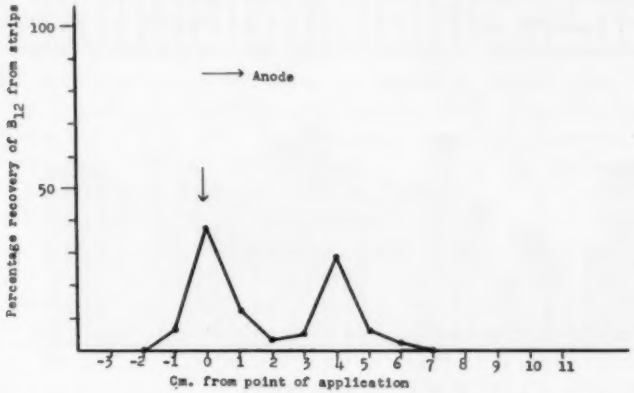

Fig. 5.—Migration of vitamin B_{12} on paper electrophoresis of rabbit liver homogenates excluding cell debris and nuclear fraction.

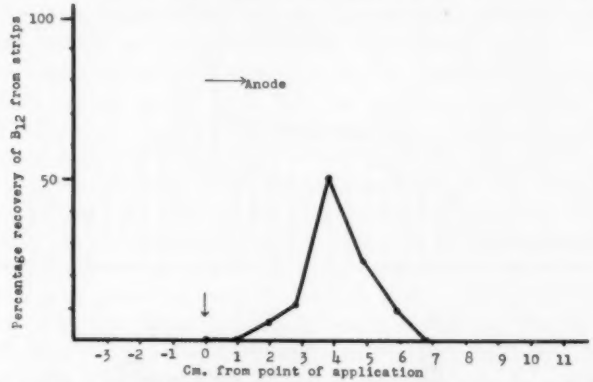

Fig. 6.—Migration of vitamin B_{12} on paper electrophoresis of supernatant fluid fraction of rabbit liver homogenates.

The decrease of urinary vitamin B_{12} excretion was concomitant with an increase in the serum vitamin B_{12} levels, and vice versa, the increase of the former corresponded to the decline of the latter. The elevation of the serum vitamin B_{12} levels after CCl_4 administration could not, of course, be interpreted simply by the retention of vitamin B_{12} in the urine. Forty-eight hours after CCl_4 administration, the increase in total serum vitamin B_{12} could be

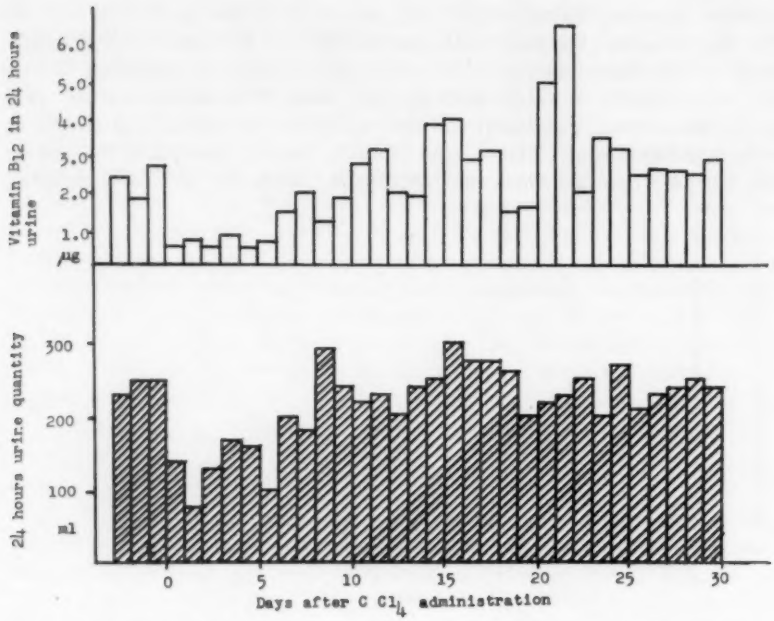

Fig. 7.—Urinary excretion of vitamin B_{12} in a rabbit given CCl_4 (Rabbit 1).

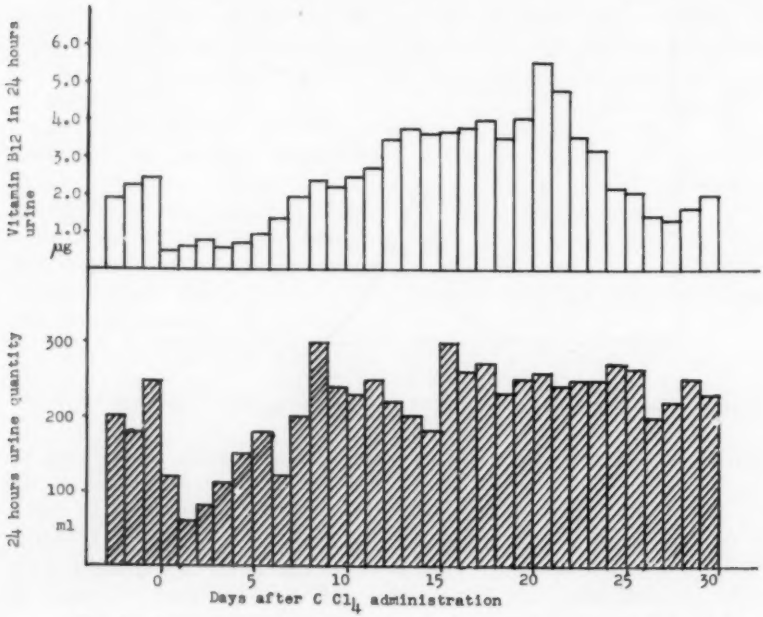

Fig. 8.—Urinary excretion of vitamin B_{12} in a rabbit given CCl_4 (Rabbit 2).

calculated to be about 16 μg, but the apparent vitamin B_{12} retention for this period was only a few micrograms. Therefore, the disturbance of the urinary excretion of vitamin B_{12} seemed to have little effect on the elevation of the serum vitamin B_{12} levels after CCl_4 administration, in contrast to the change of the vitamin B_{12} storage in the liver.

DISCUSSION

A single injection of CCl_4, in the doses described, produced markedly severe histologic damages of the liver; however, the damages were only temporary and recovery was rapid and without histopathologic change.

In the studies reported here, the following points have been clarified. (1) The elevation in the serum vitamin B_{12} levels after CCl_4 administration in rabbits has a close time relationship to the development of acute hepatic necrosis and to the decrease of the vitamin B_{12} storage in the liver. (2) The elevation is grossly proportional to the severity of the liver damages provoked by CCl_4. (3) The amount of total serum vitamin B_{12} which is increased within 48 hours after the administration of CCl_4 has a quantitative relationship to the amount of vitamin B_{12} which is decreased in the whole liver. These facts suggest strongly that the increase of the serum vitamin B_{12} levels is due to the release of storage vitamin B_{12} into the blood stream which occurs following the hepatocellular disintegration due to CCl_4 intoxication. The effect of CCl_4 is not limited, of course, only to the liver; the kidneys and the intestinal tract may also be involved. In fact, except for the liver, the kidneys may be the most important organ for the storage of vitamin B_{12}. The problem of the release of vitamin B_{12} storage from the kidneys after CCl_4 may be neglected because of the lower vitamin B_{12} content.

The bound form of serum vitamin B_{12} was much more markedly increased in rabbits given CCl_4, but the increase of the free form was less marked and temporary. Jones and associates[4] have reported that patients with acute hepatitis have large amounts of the free form of serum vitamin B_{12} but have very little increase in the amount of the bound form. The proportional amount of the free form of the serum vitamin B_{12} levels varies with the animal species.[21, 22] This may partially account for the difference in the increase between patients with acute hepatitis and rabbits with experimental hepatic necrosis. In normal human subjects, the bound form has been found in the alpha globulin fraction,[6, 7] and the free form has been recovered mainly from the beta globulin fraction by paper electrophoresis.[6] However, the free vitamin B_{12} may not be free in the true sense of the word inasmuch as it seems to have a loose binding with protein in the beta globulin fraction.[6] In normal rabbit serum the vitamin B_{12} is located mainly in the alpha and beta globulin fractions and, as shown by paper electrophoresis, the peak of greatest activity is in the alpha globulin fraction. The vitamin B_{12} increased in rabbit serum after CCl_4 administration could not be differentiated by paper electrophoresis from the vitamin existing naturally before the treatment. Vitamin B_{12} in the supernatant fluid fraction of rabbit liver homogenates might be bound to a protein which has an electrophoretic mobility

similar to the beta globulin fraction of rabbit serum. Pitney and co-workers[23] have reported that vitamin B_{12} in human liver exists as a loosely bound protein complex with an electrophoretic mobility similar to that of serum beta globulin. They have also stated that in some experiments there was a second peak of vitamin B_{12} activity at the point where the liver homogenates were applied to the paper. It is most likely, from the present studies, that the peak of vitamin B_{12} activity near the point of application is due to the vitamin B_{12} in particulate fractions, especially in the mitochondrial fraction. Vitamin B_{12} in rabbit liver is available to *L. leichmannii* as free vitamin B_{12} without preliminary heating, and this fact has been observed in the vitamin B_{12} of human liver.[23] It can be hypothesized, therefore, that the nature of the free form of serum vitamin B_{12} is identical with the vitamin present in the supernatant fluid fraction of rabbit liver cells. If this is the case, the increase of free form of serum vitamin B_{12} in patients with acute hepatitis or in rabbits with experimental hepatic necrosis is due to the release of vitamin B_{12} beta globulin complex in the damaged liver cells. But in rabbits with experimental hepatic necrosis the increase of the bound form of serum vitamin B_{12} was much more marked, and there seems to be a transfer mechanism whereby vitamin B_{12} bound to beta globulin in liver cells is carried to specific vitamin B_{12}-binding proteins in the serum.

Intracellular distribution of vitamin B_{12} in normal rabbit liver is similar to the result reported by Swendseid and associates[24] in their studies on rat liver. More than 50 per cent of the vitamin B_{12} content in rabbit liver is distributed in the mitochondrial fraction. Considering the possibility that the vitamin B_{12} present originally in mitochondria is partially released during homogenation and fractionation, more concentrated vitamin B_{12} may exist in mitochondria of the intact liver cells. Vitamin B_{12} is markedly decreased in the mitochondrial fraction of the damaged liver cells, and this fact seems to be related to findings that cytochrome c[25] and pyridine nucleotides,[26] the substances known to be concentrated in mitochondria, are also decreased after CCl_4 administration.

The 24-hour urine quantities were diminished for a week after CCl_4 administration, probably due to a toxic nephrosis which resulted from the intoxication of CCl_4, and there was a concurrent decrease in vitamin B_{12} excretion. Jones and associates[4] have reported that the amount of vitamin B_{12} in the urine appears to be closely related to the amount of free form of serum vitamin B_{12} both in normal persons and in patients with liver disease. In rabbits such a correlation was not observed, but in these cases the CCl_4-induced kidney injury was different from the injury in patients with liver diseases. Yamamoto and associates[27] studying the effect of CCl_4 injury on plasma and liver vitamin B_{12} levels in rats have reported that CCl_4 increases the serum vitamin B_{12} levels and also the urinary excretion of vitamin B_{12} of rats fed a casein diet. Their results on the urinary excretion of vitamin B_{12} are different from ours, although they did not determine the total amounts of the urinary excretion of vitamin B_{12}.

It has been reported that administration of vitamin B_{12} protects rats from the liver damages by CCl_4 intoxication to a considerable degree.[28, 29] The protective action of vitamin B_{12} seems to be conflicting with the fact that pathologic high levels of serum vitamin B_{12} result from CCl_4 intoxication. Mechanism of the protective action should be investigated from the other point of view.

SUMMARY

Serum vitamin B_{12} levels of rabbits given CCl_4 were rapidly and markedly increased, and concomitantly the vitamin B_{12} content of the liver was decreased. These have a close time relationship to each other and to the development of acute experimental hepatic necrosis. The increase in serum vitamin B_{12} levels was grossly proportional to the severity of the liver damages and had a quantitative relationship to the decrease of the vitamin B_{12} content of the liver. The increase of serum vitamin B_{12} levels was mainly due to the increase of bound form of vitamin B_{12}, and the increase of free form was less marked and temporary. Serum vitamin B_{12} levels of rabbits given CCl_4 declined after the hepatic damages were restored almost completely. The serum vitamin B_{12} of rabbits was located mainly in alpha and beta globulin fractions of serum protein, and vitamin B_{12} increased in the serum after CCl_4 administration was also located in the same fractions. Over half of the vitamin B_{12} content in normal rabbit liver cells was distributed in mitochondria; in the cells of the CCl_4-damaged liver vitamin B_{12} content in mitochondria was markedly decreased. The urinary excretion of vitamin B_{12} in rabbits was diminished for a week after CCl_4 administration due to renal damages provoked by CCl_4; thereafter it was increased gradually showing a time relationship to the decline of serum vitamin B_{12} levels. From the results of the present studies it was strongly suggested that when the liver cells are damaged and lapse into acute necrosis, storage vitamin B_{12} in the cells is rapidly released into the blood stream, and pathologic high levels of serum vitamin B_{12} occur.

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STUDIES OF ACID MUCOPOLYSACCHARIDE METABOLISM IN HUMAN SUBJECTS GIVEN RADIOACTIVE SULFATE INTRAVENOUSLY

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ACID mucopolysaccharides (APS) are widely distributed throughout the body, particularly in mesothelial tissues, but are found elsewhere and are present in serum and urine.[1-6] Although chondroitin sulfate appears to be the major APS of serum and urine,[4, 5, 7, 8] hyaluronic acid also is present in serum[6] and may occur in low concentration in urine.[9]

The concentration of APS in serum or its rate of excretion in urine has been found to be increased in patients with rheumatoid arthritis,[9, 10] lupus erythematosus,[11] cancer, leukemia, and other malignant diseases,[8, 12] and during rapid growth in childhood.[13] These states are associated with abnormal or rapid connective tissue metabolism; a fact compatible with the suggestion that the concentration of APS somehow reflects the metabolic state of APS containing tissues of the body. However, because subjects with many of these states have other features in common, such as increased concentration of mucoproteins in serum,[10, 12, 14, 15] no definite conclusion can be drawn as to the source of APS of serum and urine.

This article reports the changes in specific activity of sulfate and APS of urine after administration of tracer quantities of carrier-free radioactive sulfate (S^{35}) to human subjects.* The studies were undertaken to see if systematic differences of APS metabolism could be demonstrated between normal subjects and patients with abnormal bone metabolism. Although the observed changes were relatively minor and inconsistent, the data did yield new information as to the kinetic state of APS of serum and urine, and for this reason are presented here.

METHODS

One or two doses of between 100 and 250 μc of carrier-free sulfate S^{35} were given by vein to 5 subjects. Blood and urine samples were collected during the following 5 to 22 days. Concentration of total and inorganic sulfate in urine was determined by gravimetric analysis[16] and of total sulfate of serum by isolation as benzidine sulfate[17] and subsequent measurement of ultraviolet absorption. Urinary APS was isolated,[18] dialyzed against running tap water for 24 hours, and its glucuronic acid content determined. Particular samples collected during the first, third, and fifth days were incubated with trypsin; the trypsin was

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precipitated with trichloroacetic acid and the supernate dialyzed.[8] Nondialyzable material was then concentrated and its glucuronic acid content measured. Other samples prepared in the same way were dried over P_2O_5 and analyzed by methods described elsewhere.[5] Radioactivity of aliquots of serum, urine, and urinary APS (hydrolyzed by HCl) was determined after addition of sufficient carrier sulfate to permit precipitation of 4 mg. sulfur as $BaSO_4$.[19]

Freshly voided samples of urine from other subjects not given S^{35} were mixed with sulfate S^{35}. APS was then isolated and dialyzed against running tap water as described above, either immediately after addition of S^{35} or after 16 hours of incubation at 4° or 37° C. The concentrations of glucuronic acid and radioactivity of the APS were then measured.

The radioactivity of each sample (urine, APS, or serum) was compared to that of a standard equal to a known fraction of the administered dose of S^{35}. The concentration of sulfate in the same samples was measured or calculated as described below and the specific activity expressed as per cent of the administered dose per gram of sulfur. The sulfur concentration of urine was determined by individual analysis of each sample. The concentration of sulfur of individual samples of APS of urine was calculated from the measured concentration of glucuronic acid by use of the assumption that sulfur and glucuronic acid were present in equimolar quantities. This assumption is justified by theoretical considerations, by results of analysis of other samples of urinary APS,[5, 8] and from analysis of samples from two of the experiments reported in this study (molar ratios of sulfur to glucuronic acid of 1.0 and 1.3). The sulfate concentration of a sample of pooled serum was measured. Because serum sulfate concentration is relatively constant, and because the fall of S^{35} concentration was so great that small changes of sulfate concentration would make relatively little difference to the specific activity curve (Fig. 1), it was assumed, for the purpose of calculating specific activity, that the serum sulfate concentration did not change significantly during the experiment. The specific activities of serum and urine sulfate and urinary APS were plotted on semilogarithmic ordinates against the time of the mid-point of each collection period. It was found that the resulting die-away curves for specific activity could be decomposed by conventional graphic methods into three exponential rates.

RESULTS

Fig. 1 presents the rates of fall of specific activity of serum and urine sulfate and of isolated urinary APS sulfate from a normal female patient given a dose of carrier-free S^{35}. The measured concentration of sulfate in serum was 3.1 mg. per 100 ml. In this and one other subject, the specific activity both of total and of inorganic sulfate of each sample of urine was determined. No significant difference was observed between the specific activity of total and inorganic sulfate, an expected result because of the small amount of organic sulfate present. In other studies, specific activity of total sulfate only was calculated.

It can be seen that specific activity of sulfate of blood and urine fell at similar extremely rapid rates, but that the specific activity of urinary APS fell much more slowly. An indication of the variation between patients can be found in Table I where the specific activities of the sulfate and APS on the fifth day are compared, and in Fig. 2 where the average values and standard deviations of sulfate and APS of urine are shown. In every case the specific activity (sulfate or APS) fell by a single exponential rate from the fourth day until the end of the study (in one subject for 22 days). Important clinical data and the average daily rate of excretion of APS (based on analysis of at least

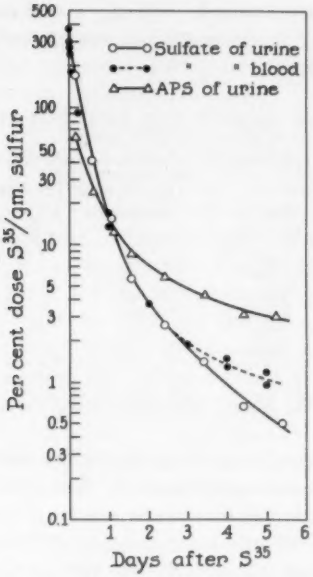

Fig. 1.—Specific activity of serum and urine sulfate and urinary acid mucopolysaccharide after intravenous administration of S^{35}.

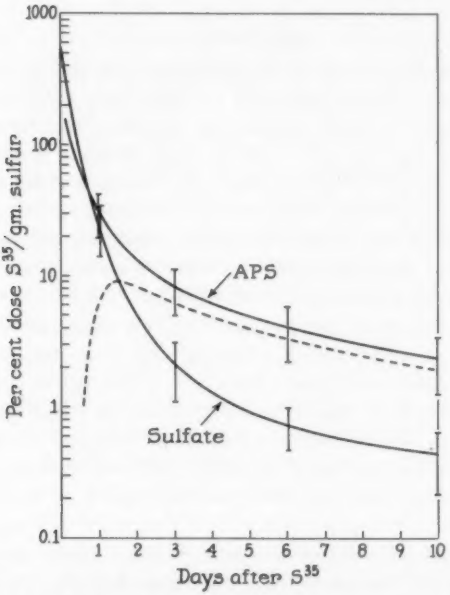

Fig. 2.—Average of specific activities of urinary acid mucopolysaccharide and sulfate for 7 tests in 5 subjects given S^{35} intravenously. The vertical bars show ± S.D. The dashed line represents difference between specific activity of APS and urinary sulfate.

two 24-hour urine collections for each subject) are shown in Table I. APS excretion rates were normal[18] in every subject, except for one with osteoporosis in whom it was consistently low.

TABLE I. CLINICAL DATA AND SPECIFIC ACTIVITY OF URINE SULFATE AND APS ON THE FIFTH DAY AFTER S^{35} WAS GIVEN

DIAGNOSIS	SEX	AGE	DOSE OF S^{35} (μc)	APS EXCRETION (MG./DAY OF GLUCURONIC ACID)	SPECIFIC ACTIVITY 5 DAYS AFTER S^{35} (% S^{35}/GM. S)	
					URINE SULFATE	APS
Normal	F	25	100	4.2	0.90	8.7
Normal*	F	37	216	4.5	0.57	2.9
Normal	F	24	107	6.7	1.28	4.0
Hyperthyroid†	F	24	100	5.5	0.52	3.6
Osteoporosis	F	69	250	1.6	0.57	7.2
Paget's disease	M	56	250	5.6	1.13	4.3

*Subject of study presented in Fig. 1.

†The second study was started on the ninth day of a 28-day course of treatment with l-triiodothyronine, 0.150 mg. daily by mouth. The two doses of S^{35} were given 60 days apart.

The ratios of concentration of glucuronic acid to radioactivity of preparations of APS were not changed significantly by incubation with trypsin. The samples had the characteristic metachromatic staining reaction and chromatographic mobility previously described for urinary APS and similar to that of chondroitin sulfate.[8] The specific activity of APS isolated from urine after in vitro incubation with sulfate S^{35} was never more than 0.008 per cent of the specific activity of sulfate isolated from the same sample of urine.

DISCUSSION

Although various aspects of APS metabolism are poorly understood, some features may be noted. When expressed as glucuronic acid, the concentration of APS in normal serum is about 2.8 mg. per liter[4] and the rate of excretion in urine from 2.7 to 7.3 mg. per day.[18] Most APS of urine is excreted from the blood, a fact shown by direct observation[20] and borne out by the finding that abnormal rates of excretion occur in generalized states rather than in urinary tract disease. Although the mechanism of its excretion has not been studied, it has been shown that increased rates of excretion occur in states known to be associated with increased concentrations of APS in serum.[5, 9, 10, 16]

The subjects of this study were chosen to allow comparison of excretion of S^{35} by normal subjects with that of patients with bone diseases characterized by abnormal rates of bone formation. Relatively small differences were observed between the rates of fall of specific activity of urinary sulfate or of APS of these various subjects. The specific activity of sulfate and APS of one subject fell more rapidly when she was made hyperthyroid than during a control period. This finding is consistent with the general acceleration of metabolic processes during thyrotoxicosis.

The specific activity of sulfate of serum was similar to that of urine until the fourth day after S^{35} was given and then exceeded that of urine (Fig. 1). By this time the specific activity of inorganic sulfate had dropped to about 1

per cent of its highest value. It is presumed that the specific activity of inorganic sulfate of blood and urine remained equal, and that the extra concentration of S^{35} measured in the blood during the fourth and fifth days was a contribution from organic compounds containing S^{35}.

The urinary acid mucopolysaccharide was found to contain appreciable amounts of S^{35} even during the first 9 hours after S^{35} was given. This did not appear to be present as a result of absorption, inasmuch as the radioactivity of the APS was not changed by incubation with trypsin and dialysis. Furthermore, APS of urine incubated in vitro with S^{35} was essentially free of S^{35} after dialysis. The facts that the specific activities of APS of the first urine samples collected (usually from 0 to 9 hours; in one subject from 0 to 4 hours after administration of S^{35}) averaged 48 per cent of those of sulfate of the same urines, and that the average fall of specific activity of APS during the next 24 hours was to 22 per cent of its highest level, suggest that part of the APS of urine is very rapidly synthesized and excreted. It has been shown that some gastrointestinal mucins have such rapid turnover rates.[21] The data presented here do not show whether the high early specific activity of APS and its subsequent rapid fall are the result of excretion of some rapidly metabolized fraction of APS of serum or of contamination of the samples with mucins from the lower urinary tract. However, in either case, it is probable that the APS responsible for this high early specific activity; because of its rapid turnover, would continue to reflect the specific activity of inorganic sulfate. To the extent that this is so, an approximate estimation of the specific activity of other sulfated APS of serum can be obtained by subtracting the specific activity of sulfate from that of APS (shown by the dashed line of Fig. 2). This suggests that after 48 hours, the specific activity of such APS fell at a single exponential rate. This rate varied from subject to subject (half time of from 3.0 to 11.0 days for individual patients and of 5.3 days for the averaged data charted in Fig. 2) with a mean of 13 per cent per day. Reported half times for chondroitin sulfate isolated from skin and cartilage of adult rats and rabbits are from 7.7 to 17 days.[22-24] No similar studies have been carried out in man, but because metabolic processes of small mammals are generally more rapid than those of human beings, even slower rates might be expected. The turnover rates of APS of serum indicated by this study are comparable to those of serum globulins of human subjects.[25]

SUMMARY AND CONCLUSIONS

1. The specific activity of the total sulfate of serum and urine and of acid mucopolysaccharides of urine was measured during the first 5 to 22 days after intravenous administration of sulfate S^{35} in 7 tests of 5 adult human subjects.

2. Specific activities of serum and urinary sulfate fell precipitously to low values by somewhat similar rates for all subjects studied.

3. The specific activity of acid mucopolysaccharide, at first lower than that of sulfate, fell less rapidly so that it became equal to and then exceeded that of sulfate after the first day.

4. The average half time of serum acid mucopolysaccharides was calculated to be about 5.3 days.

I wish to acknowledge the assistance of Mr. James Ievolella.

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SODIUM EXCRETION IN RENAL DISEASE

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THE control of sodium excretion ultimately depends on the kidney, whether this is due primarily to changes in filtration rate[1] or tubular reabsorption.[2] Patients with renal disease often show abnormalities in the way in which they handle sodium[3] that might help in understanding sodium excretion. This study was undertaken to investigate (1) the relation between inulin and sodium clearances in patients with renal impairment and (2) the effect of sodium loading on the inulin and sodium clearances in patients with renal disease.

METHODS

Patients with renal disease were selected on the basis of an abnormal urinary sediment, and/or proteinuria and histologic confirmation by means of needle biopsy or autopsy. The degree of renal disease varied from terminal renal insufficiency to little or no functional impairment.

Needle biopsies of the kidneys were done in all patients with the exception of 14 who were considered as normal controls. Specimens were fixed, embedded, sectioned, and stained with hematoxylin and eosin and by the periodic acid-Schiff technique as previously reported.[4] Usually in 3 to 4 days tests of renal functions were performed.[5, 6] Patients were maintained on a regular hospital diet containing about 5 Gm. of sodium chloride per day. Prior to the measurement of renal function, patients were fasted for 12 hours. At 7:00 A.M. on the morning of the tests, 200 ml. of tap water was given by mouth. At 8:00 to 8:30 A.M. a priming dose of inulin was given and an infusion containing inulin and sodium p-aminohippuric acid (PAH) was started. Thirty minutes later the bladder was flushed with sterile distilled water and air. Venous blood samples were obtained through an indwelling needle at the mid-point of alternate urine collection periods. Inulin was determined by the method of Roe and associates,[7] PAH by the method of Smith[5] and sodium was measured using a Barclay flame photometer with an internal lithium standard. Determinations were done in duplicate.

After 3 control periods, those patients who were chosen for sodium loading received 100 ml. of 5 per cent NaCl rapidly intravenously (3 to 5 minutes) and specimens were obtained for 3 additional collection periods. Some patients then received no further NaCl loads. Others were subjected to one or two additional infusions of 100 ml. of 5 per cent NaCl, and the collection periods were continued. In those patients receiving 1 or 2 infusions of NaCl, 3 urine collection periods and 2 venous blood samples were obtained each time. In those who received 3 separate infusions of NaCl only 2 urine collection periods and 2 venous blood samples were obtained after each infusion.

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RESULTS

Sodium and inulin clearances were determined in 44 patients with renal disease proved by renal needle biopsy or autopsy (Table I). It was found (Fig. 1) that there was rough correlation between these two clearances ($r = 0.7307$). In 17 convalescent individuals who had no evidence of renal disease

TABLE I

	NUMBER
Nephrosclerosis	4
Acute glomerulonephritis	14
Chronic glomerulonephritis	5
Tubular necrosis	5
Chronic pyelonephritis	3
Membranous glomerulonephritis	9
Amyloidosis	1
Myeloma kidney	1
Renal vein obstruction*	1
Milk-alkali syndrome*	1
Total	44
Normal	17
Total	61

*Basement membrane thickening in the glomeruli was the lesion seen.

either by renal biopsy (3 patients) or clinical findings (14 patients), no such relation was seen (Fig. 2). Results from patients with heart failure or from those who were within 24 hours of death were excluded. Edema, not considered due to heart failure, was found in 2 patients with acute glomerulonephritis, 2 with membranous glomerulonephritis, 5 with chronic glomerulonephritis, and one each with renal vein thrombosis and amyloidosis. There was no significant correlation in any of the groups between sodium excretion and the filtration fraction (Fig. 3). These findings suggested that in renal disease there was a relationship between sodium excretion and glomerular filtration. In order to test this further the second part of the study was done. Sixteen patients were studied by means of sodium loading. Sixteen received 100 ml. of 5 per cent NaCl, 9 received an additional 100 ml. of 5 per cent NaCl, and 4 received a third infusion of 100 ml. of 5 per cent NaCl. The results are shown in Table II.

In calculating the difference from the mean in the clearance values for each patient for the control periods a variation of 7.6 ± 10.1 ml. per minute was found in inulin clearance and 0.045 ± 0.039 ml. per minute in sodium clearance. Duplicate serum samples gave differences in serum sodium values of 0.66 per cent. A change in sodium clearance was considered significant when the value changed by more than 3 times the mean deviation (0.045 ml. per minute) of the control value. Using these criteria 10 patients out of 16 showed an increase in sodium clearance following 100 ml. of 5 per cent NaCl, 8 patients out of 9 showed an increase after an additional 100 ml. of 5 per cent NaCl, and 3 patients out of 4 after a third infusion of 100 ml. of 5 per cent NaCl (Table II).

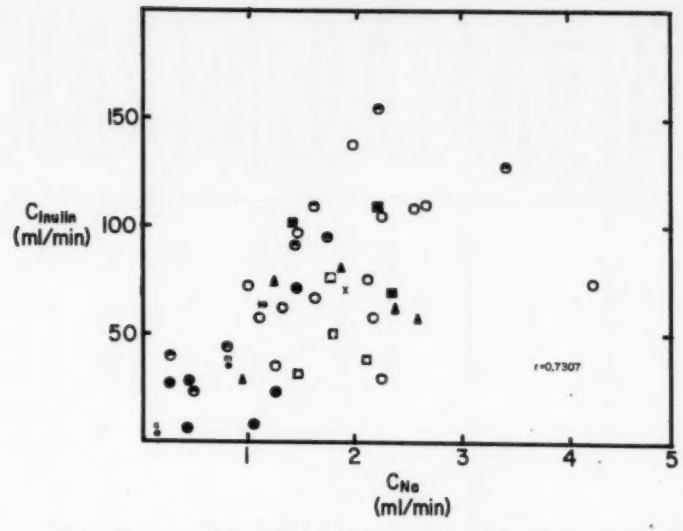

Fig. 1.—The relation between sodium and inulin clearance in patients with renal disease. Key: ■ = nephrosclerosis; ○ = acute glomerulonephritis; ● = chronic glomerulonephritis; ▲ = tubular necrosis; □ = chronic pyelonephritis; ◒ = membranous glomerulonephritis; a = amyloidosis; m = myeloma kidney; †● = renal vein obstruction; x = milk-alkali syndrome.

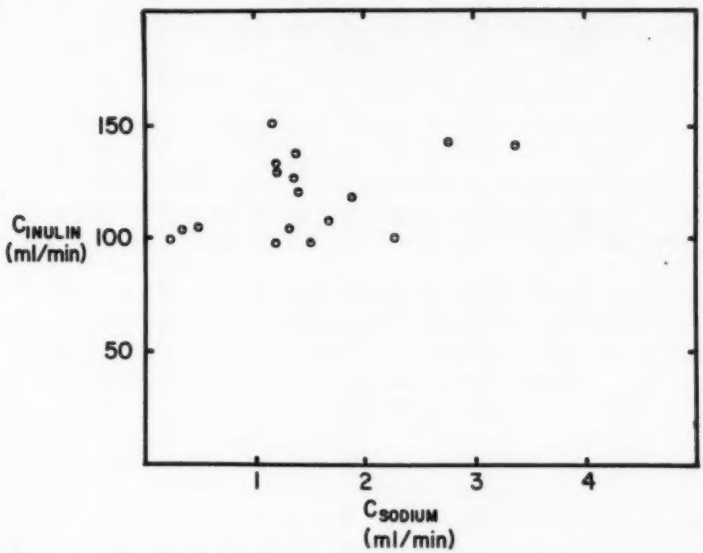

Fig. 2.—The relation between sodium and inulin clearance in patients without renal disease.

TABLE II

PATIENT	CLEARANCE INULIN				FILTERED Na*				EXCRETED Na*				CLEARANCE Na†				TISSUE DIAGNOSIS
	CONTROL‡	100§	200§	300§	CONTROL	100	200	300	CONTROL	100	200	300	CONTROL	100	200	300	
1	9	11	–	–	1.22	1.52	–	–	0.0334	0.0549	–	–	0.26	0.42	–	–	Lupus erythematosus
2	8	9	–	–	1.07	1.25	–	–	0.144	0.103	–	–	1.00	0.68	–	–	Chronic pyelonephritis
3	26	34	–	–	3.49	4.55	–	–	0.195	0.487	–	–	1.18	2.86	–	–	Pyelonephritis and intercapillary glomerulosclerosis
4	155	88	–	–	20.8	11.9	–	–	0.337	0.418	–	–	1.75	3.25	–	–	Nephrosclerosis
5	3	3	–	–	0.39	0.405	–	–	0.086	0.136	–	–	0.63	0.98	–	–	Membranous glomerulonephritis
6	4	4	–	–	0.545	0.556	–	–	0.0342	0.0514	–	–	0.27	0.37	–	–	Membranous glomerulonephritis
7	30	32	35	–	3.52	3.75	4.20	–	0.0151	0.0219	0.0223	–	0.11	0.16	0.16	–	Chronic glomerulonephritis
8	39	33	30	–	5.24	4.45	4.08	–	0.214	0.266	0.289	–	1.72	2.13	2.30	–	Nephrosclerosis
9	4	5	5	–	0.52	0.67	0.67	–	0.092	0.142	0.183	–	0.59	0.76	1.22	–	Nephrosclerosis and pyelonephritis
10	63	59	98	–	8.38	7.80	13.2	–	0.0365	0.126	0.376	–	0.82	0.97	2.84	–	Intercapillary glomerulosclerosis
11	101	88	102	–	14.0	12.5	15.4	–	0.0999	0.112	0.123	–	0.61	0.73	0.76	–	Normal
12	34	32	33	–	4.63	4.45	4.60	–	0.368	0.412	0.467	–	2.66	2.61	3.32	–	Acute and chronic pyelonephritis, sarcoidosis
13	116	90	72	80	15.9	12.6	10.2	11.4	0.214	0.248	0.323	0.432	1.48	1.66	2.16	2.86	Lupus erythematosus
14	18	20	24	26	2.32	2.64	3.17	3.38	0.0296	0.0276	0.0337	0.0343	0.23	0.20	0.24	0.24	Chronic glomerulonephritis
15	110	87	132	86	15.3	13.3	19.3	13.0	0.287	0.354	0.495	0.434	1.90	2.27	3.12	2.65	Membranous glomerulonephritis
16	54	60	117	85	7.03	7.80	15.9	11.2	0.112	0.144	0.310	0.318	0.84	1.08	1.73	2.34	Membranous glomerulonephritis

*Milliequivalents per minute.
†Milliliters per minute.
‡Average of 3 values.
§Average of 2 values.

Three patients had a rise in inulin clearance exceeding 3 times the mean deviation of the control. In all 3 patients this occurred following the second infusion of NaCl. There was a drop in inulin clearance in 4 patients (Subjects 4, 11, 13, and 15); however, in one of these patients (Subject 15) the inulin clearance again rose. All of these patients had relatively high inulin clearance values during the control period. One had no kidney disease, and one was recovering from acute renal disease.

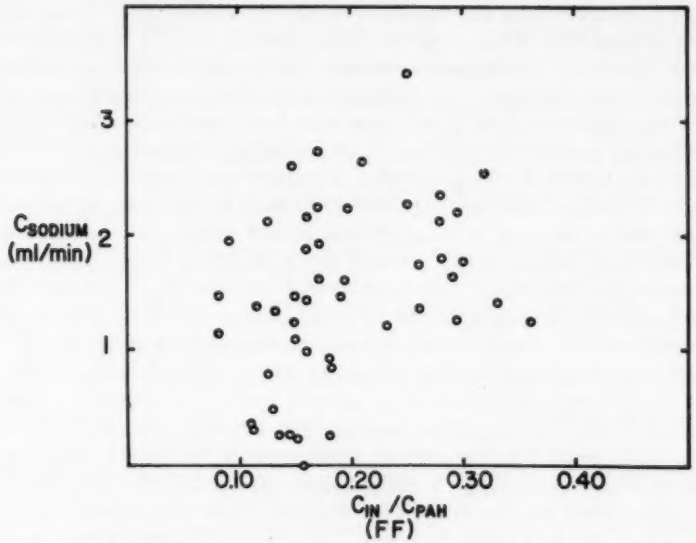

Fig. 3.—The relation between sodium clearance and filtration fraction in patients with renal disease.

Excreted sodium (Table II) increased in all but one patient. This did not parallel filtered sodium in 6 instances, although it agreed with the sodium clearance in all but one patient. The per cent of filtered sodium excreted rose in all but 4 patients.

Serum sodium rose to some degree in all but 1 patient and fell in none.

In these instances it would appear that the change in sodium clearance was not always related to an increased inulin clearance. In fact, sodium clearance increased in 3 patients whose inulin clearance decreased.

DISCUSSION

The problem of sodium excretion has been the subject of several excellent reviews.[1, 8, 9] Isotonic sodium loading in normal man has produced varying results. Sodium excretion is increased[10-12] while filtration rate may or may not rise.[11] Hypertonic sodium loading increases sodium excretion and usually filtration rate[11, 13-16] although in some[17] there is no reported change. Many of these changes can be explained by an increase in filtered sodium since both

serum Na^+ and glomerular filtration rise. Oral loading with sodium chloride increases the filtration rate but a decrease in the tubular reabsorption of sodium seems also to play a part.[18] Recent evidence has indicated that an increase in the colloid osmotic pressure in the efferent arteriole plays an important role in sodium retention in heart failure.[19, 20] This is accompanied by a rise in the filtration fraction. This does not seem to hold true in the patients studied without heart failure but with renal disease. Nickel and associates[21] have demonstrated an increased loss of sodium in renal disease. Sodium deprivation in these patients resulted in a decreased glomerular filtration rate. From our data it would appear that in patients with renal disease there is a correlation between inulin clearance (reflecting glomerular disease) and sodium clearance suggesting that under ordinary circumstances these two are closely related. This suggests that filtered sodium is the more important element in determining sodium excretion although other factors (such as tubular reabsorption or lack thereof) cannot be excluded. If glomerular filtration were relatively "fixed" by disease and if tubular function were merely passive in its effect on sodium excretion, then sodium loading in renal disease should produce an increase in sodium excretion only insofar as it increased serum sodium. Under conditions of sodium loading in this study, this relation did not always hold true. In only 3 patients did inulin clearance rise whereas sodium clearance rose in 11 patients. Because of the rise in serum sodium, filtered sodium increased in 9 patients; however, there were several instances where filtered sodium either fell or failed to rise even though excreted sodium and sodium clearance rose (Subjects 4, 5, 8, 9, 12, and 13). This suggests that although sodium excretion usually seems to be related to the rate at which sodium is filtered, there are instances where a decreased tubular reabsorption of sodium also appears to play a role. This could be due to the fact that the renal tubules are unable to reabsorb sodium, i.e., a T_m Na^+ had been reached or that the tubules exert some degree of control over sodium excretion and allow excess sodium to be excreted. Previous work[21] has also shown that sodium loading in renal disease increases sodium excretion out of proportion to the glomerular filtration rate.

If the former were true these patients would be expected to show greater histologic changes in the tubules than the rest of the patients. This did not seem to be true. There does not seem to be a relation between the type of renal pathology present and the ability to handle sodium.

SUMMARY

1. In 44 patients with renal disease proved by autopsy or biopsy sodium excretion seemed dependent on filtered sodium.

2. Sodium loading usually produced an increased filtered load in patients with renal disease, but in some instances excreted sodium increased even without an increase in filtered sodium, suggesting a lack of tubular reabsorption under these circumstances.

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RENAL FUNCTION IN HUMAN PREGNANCY

II. Effects of Venous Pooling on Renal Hemodynamics and Water, Electrolyte, and Aldosterone Excretion During Normal Gestation

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NUMEROUS investigators have shown that venous pooling produced by either quiet standing or venous occlusion of the lower extremities causes a striking decrease in the excretion of water and electrolytes. Since ethyl alcohol, which presumably interferes with the release of antidiuretic hormone (ADH) by the neurohypophysis,[1] inhibits the antidiuresis of quiet standing,[1-3] it has been assumed that a major factor in this type of oliguria is an increased elaboration of ADH. The antinatriuresis has been attributed in part by some authors[4, 5] to the changes in renal hemodynamics, while others[2, 6, 7] have considered these changes too small to account for it.

The role of aldosterone has not been well defined; however, the higher aldosterone output during the day than during the night[8, 9] is attributed by Muller and his co-workers[8] to the upright position. It is generally agreed that several clinical and experimental circumstances associated with increased aldosterone output have in common either hypovolemia or redistribution of the intravascular volume from the arterial to the venous side of the circulation.[10-13]

Since pregnant women have increased tendency to venous pooling in the lower extremities owing to the increased intra-abdominal pressure and the pressure of the heavy uterus on the major vessels, we decided to investigate the effects of this pooling on renal hemodynamics and water, electrolyte, and aldosterone excretion throughout the course of normal gestation. This problem becomes pertinent since pregnant patients commonly accumulate a significant amount of edema in the lower extremities during the day which may disappear at night or on bed rest. Therefore it was hoped that the present studies might provide some information regarding the mechanism of this type of edema and at the same time might contribute further to the understanding of renal handling of water and electrolytes during venous pooling.

MATERIALS AND METHODS

The studies were carried out on 22 normally pregnant subjects who had no previous history of cardiovascular or renal diseases and who were divided into 4 groups.

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The first group (Group I) comprised 7 subjects who volunteered for the study of the effects of quiet standing. These studies were performed in each of the 7 patients once in the first trimester, once in the second trimester, and once in the third trimester of pregnancy and were repeated again 6 to 7 weeks postpartum (2 patients missed the postpartum test). The test was carried out in the hospital in a separate and quiet room with the patient lying in bed most of the time in the supine position.* On the morning of the test, breakfast was omitted and the patient was given an oral water load of 1,000 ml., followed by a constant infusion of 5 per cent glucose in water aimed at maintaining a positive balance of about 1 liter. A sufficient amount of insulin was added to the infusion to prevent glycosuria. Clearances of inulin and para-aminohippurate (PAH) were obtained by a technique previously described[15] with 30 to 60 minutes of equilibration after the priming injection. Blood samples were collected at the mid-point of each period and urine was collected by means of an indwelling catheter in the bladder and air washouts. Three to four clearances, each of 30 minutes' duration, were collected in the lying position and 4 similar clearances during quiet standing. Several patients exhibited syncope or near-syncope while they were standing and were therefore permitted to sit or lie down for a brief period of time. Although this did not seem to alter materially the renal effects of quiet standing, the severity of the side effects prevented us from prolonging these studies through recovery periods.

The second group (Group II) consisted of 5 patients who were in the last trimester of gestation and who served for the limb occlusion experiment. The subjects remained recumbent throughout the entire study. Two specially designed inflatable cuffs were fitted around the upper thighs and connected to an automatic occluding device which uses Freon as the inflating gas.† After obtaining 3 to 4 control clearances, the 2 cuffs were instantaneously inflated to a pressure close to the diastolic pressure and 4 to 5 clearances of 20 to 30 minutes each were then collected. The techniques of hydrating the patient and of collecting the blood and urine samples were the same as of the previous group, with the exception that the rate of water diuresis induced in these patients was higher.

The third group (Group III) consisted of 7 subjects in the last trimester of pregnancy who volunteered for the alcohol experiment. Of these 7 subjects, 5 received alcohol orally and 2 intravenously. Three to four clearances of 30 minutes' duration were obtained in the recumbent position. Then, while still recumbent, each patient ingested slowly over a period of 15 to 20 minutes from 100 to 180 ml. of 86 proof bourbon or scotch whiskey and 10 to 15 minutes later she stood up and 4 to 5 clearances were obtained in the standing position. Hydration and clearance techniques were the same as Group II. Blood levels of alcohol were determined during each of these clearance periods. In 2 patients, 115 ml. of 10 per cent alcohol was given intravenously over a period of 15 minutes prior to standing and 4 clearances were collected in the standing position.

The fourth group (Group IV) consisted of 3 pregnant subjects (last trimester) on whom urinary aldosterone was determined under the following condition:

Daily sodium intake was limited to 4 Gm. of which 2 Gm. was divided into the 3 daily meals and the other 2 given at night in the form of tablets of sodium chloride. Since assay of this hormone required large aliquots of urine which the acute clearance technique could not provide, and because of a possible diurnal variation in the excretion of aldosterone, control determinations were made on 2 patients during 12-hour periods of recumbency followed by 12 hours of ambulation. In the third patient, the periods of recumbency and ambulation were reduced to 6 hours. Urine was collected and immediately acidified to pH 3 and covered with 15 to 20 ml. of chloroform. The output of aldosterone, sodium, and potassium were thus computed for each of the 12- or the 6-hour periods.

*In several subjects this position by itself produced oliguria, hypotension, and subjective manifestations of imminent collapse similar to that reported by others[16] and, therefore, the lateral position had to be used. When in a given case the latter position did not correct the oliguria, the test was discontinued and the patient was excluded from the studies.

†Robbins Instrument Corporation, Attleboro, Mass.

Blood and urine samples were analyzed for inulin, para-aminohippurate, sodium, chloride, potassium, and osmolality by methods previously described.[15, 16] Blood alcohol levels were analyzed by the method of Gibson and Blotner.[17] Osmolal and free water clearances were calculated according to the method of Smith.[18] Aldosterone in the urine was assayed by the method of Nowaczynski.[19] Blood pressure (sphygmomanometer) and pulse rate were recorded at frequent intervals throughout the studies. Renal plasma flow (RPF) was estimated from the PAH clearance and glomerular filtration rate (GFR) from the inulin clearance.

RESULTS

Group I. Quiet Standing.—

A. Systemic effects: A total of 26 tests on the effects of quiet standing were completed on the 7 subjects studied throughout pregnancy and postpartum.

The assumption of the upright position by the pregnant subject produced dizziness, fainting, and hypotension which occurred in the majority of instances within the first 20 minutes after standing. The fall in systolic blood pressure varied from 10 to 40 mm. Hg and 10 to 30 mm. Hg for diastolic pressure. In some patients, the pulse became extremely weak and tachycardic whereas in others a bradycardia was noted. Nausea was observed in approximately 50 per cent of the tests and actual vomiting occurred in approximately 10 per cent. These side effects were more severe after the sixth month of gestation, but were less frequent in the postpartum period.

B. Renal effects: Table I presents the data on renal hemodynamics and water and electrolyte excretion obtained from each test carried out throughout the course of gestation and in the postpartum period. The averages of the last 3 control and of the last 3 collection periods after standing are listed. The percentage of fall in urine flow, glomerular filtration rate, renal plasma flow, and sodium excretion for each patient is also indicated in order to facilitate comparison of the response to standing. Fig. 1 illustrates a complete experiment performed on a patient in the last trimester of pregnancy. Urine flow fell moderately within the first clearance period, but in the second, third, and fourth periods it fell sharply in every instance.

Glomerular filtration rate and renal plasma flow showed more variation after standing than urine flow. In general, no significant change or a moderate fall occurred in the first period, with a further fall in the two subsequent periods. In the last period, both glomerular filtration rate and renal plasma flow began to rise slightly but the values were still below those of the control periods. Despite the variation, if the first clearance after standing was ignored because of a possible error from the rapidly changing urine flow and the influence of the dead space, and if the second, third, and fourth clearances were averaged to determine the response to standing, then both glomerular filtration rate and renal plasma flow showed a significant decrease in all tests performed during pregnancy (Table I).

The pattern of changes in the excretion of sodium and chloride paralleled closely that of urine flow. A marked fall occurred after standing in every case (Table I and Fig. 1).

The excretion of potassium varied somewhat from one patient to another and in the same patient from one test to another. However, in general, it decreased after standing, although the fall was proportionately less than that of sodium and chloride (Table I and Fig. 1).

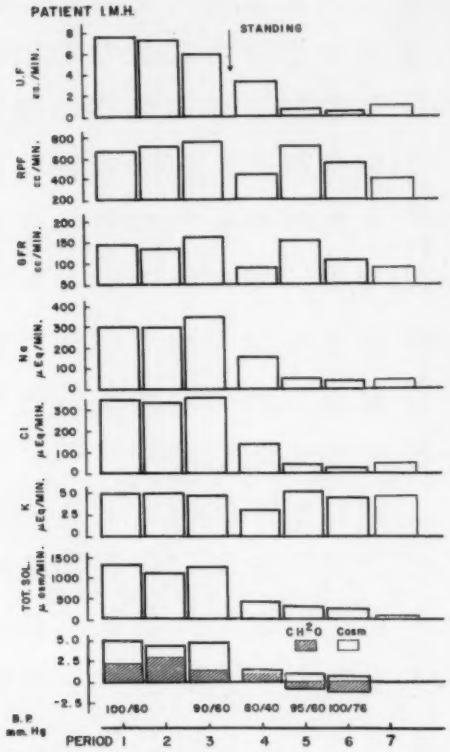

Fig. 1.—The effects of quiet standing on urine flow (UF), renal plasma flow (RPF), glomerular filtration rate (GFR), the excretions of Na, Cl, K, and total solutes, and on the free water (CH_2O) and osmolal (Cosm) clearances during the last trimester of gestation. Note the marked fall in urine flow during standing. In this patient, RPF and GFR decreased in the first period after standing, rose in the second period, but fell again in the 2 subsequent periods. The pattern of changes in excretions of Na, Cl, and total solutes paralleled that of urine flow. The excretion of K fell in the first period but returned to near control values on subsequent periods. Free water clearance fell considerably and became negative in the last 2 periods. Osmolal clearance also decreased.

The changes in total solute excretion paralleled those of urine flow and electrolytes decreasing moderately in the first period after standing with a further fall in subsequent periods (Fig. 1). Plasma osmolality varied in the control periods between 270 and 279 mOsm. per liter. It did not change significantly after standing. Plasma electrolytes also remained the same. The control values for the free water clearance (CH_2O) varied from one patient to another according to the magnitude of urine flow, but they were usually

TABLE I. DATA ON URINE FLOW, GLOMERULAR FILTRATION RATE, RENAL PLASMA FLOW, Na, Cl, K, AND TOTAL SOLUTE EXCRETIONS, AND OSMOLAL AND FREE WATER CLEARANCES OBTAINED FROM THE TESTS OF QUIET STANDING IN THE FIRST, SECOND, AND THIRD TRIMESTERS OF GESTATION AND IN THE POSTPARTUM PERIOD*

PERIOD	PATIENT	UF		GFR		RPF		Na		Cl		K		TOTAL SOLUTE		COSM		CH_2O	
		C	A	C	A	C	A	C	A	C	A	C	A	C	A	C	A	C	A
First trimester	B. M.	6.1	3.7 (39)	104	88 (15)	574	482 (16)	181	115 (30)	108	75	37	30	619	412	2.2	1.4	3.9	2.3
	S. H.	5.5	3.5 (57)	133	106 (20)	560	428 (23)	425	292 (30)	310	225	50	56	1,000	406	3.5	1.4	2.0	2.1
	J. A.	4.5	3.1 (31)	137	104 (24)	730	600 (18)	160	110 (31)	114	47	51	52	750	413	2.7	1.4	1.8	1.7
	V. J.	7.2	3.6 (50)	117	85 (27)	677	507 (25)	168	110 (35)	128	92	38	39	680	430	2.4	1.5	4.8	2.1
	I. M. H.	7.6	2.9 (62)	153	122 (20)	710	610 (14)	299	165 (45)	245	149	52	44	1,055	474	3.7	1.7	3.5	1.2
	L. H.	11.4	3.4 (70)	182	111 (33)	650	454 (30)	135	85 (37)	117	74	59	46	880	532	3.2	1.4	8.2	2.1
	B. F.	9.4	4.9 (48)	137	119 (13)	575	498 (13)	168	119 (29)	142	98	48	45	692	414	2.5	1.5	6.9	3.4
Second trimester	B. M.	5.4	1.6 (70)	168	135 (20)	649	494 (24)	130	65 (50)	120	54	48	45	701	599	2.5	2.1	2.9	-1.1
	S. H.	5.8	1.1 (81)	149	107 (28)	860	645 (25)	204	97 (52)	115	78	65	40	850	417	—	—	—	—
	J. A.	6.7	2.1 (69)	162	113 (30)	750	563 (25)	136	55 (60)	125	44	60	55	881	374	3.2	1.3	2.6	0.0
	V. J.	6.5	1.6 (75)	150	90 (40)	871	615 (29)	215	84 (61)	163	66	29	32	750	457	2.7	1.6	3.8	-0.4
	I. M. H.	8.3	1.3 (84)	150	115 (23)	730	600 (18)	390	117 (70)	288	97	59	34	1,297	648	4.5	2.2	3.8	-0.9
	L. H.	10.0	1.8 (82)	196	118 (40)	830	593 (29)	225	114 (49)	192	96	49	35	1,058	467	3.8	1.7	6.2	0.2
	B. F.	11.0	4.2 (62)	144	115 (20)	622	511 (18)	256	148 (42)	209	117	56	39	950	536	3.4	1.9	7.6	2.3

Third trimester	B. M.	7.1	1.3 (82)	160	110 (31)	680	434 (36)	126	50 (60)	113	30	47	29	588	357	2.1	1.3	5.0	0.3
	S. H.	11.2	1.3 (88)	195	98 (50)	1,005	474 (53)	139	48 (65)	85	33	101	47	1,122	481	4.0	1.7	7.2	-0.4
	J. A.	10.0	1.1 (89)	154	88 (43)	790	396 (50)	118	20 (83)	68	12	68	38	580	338	2.1	1.2	8.1	-0.2
	V. J.	6.4	0.9 (86)	165	83 (50)	830	450 (46)	225	75 (67)	163	63	32	25	480	372	1.7	1.3	4.7	-0.4
	I. M. H.	7.5	0.9 (88)	154	113 (27)	745	532 (29)	315	44 (86)	355	39	49	50	1,325	352	4.9	1.4	2.6	-0.4
	L. H.	8.7	0.9 (90)	210	106 (49)	910	587 (35)	185	77 (58)	125	54	65	38	1,097	333	4.0	1.2	4.7	-0.3
	B. F.	12.3	1.3 (89)	163	79 (51)	647	442 (32)	286	104 (64)	225	83	55	38	848	344	3.0	1.2	9.3	0.1
Postpartum	B. M.	5.7	4.3 (25)	110	110 (0)	525	543 (+3)	125	95 (24)	110	81	24	24	560	510	2.0	1.9	3.7	2.9
	S. H.	8.5	6.0 (29)	113	94 (17)	545	441 (19)	142	107 (25)	95	85	65	56	750	598	2.6	2.1	5.9	4.3
	J. A.	9.2	6.1 (33)	147	154 (+5)	657	634 (3)	195	125 (36)	165	128	55	43	820	516	2.9	1.8	6.3	4.3
	I. M. H.	9.4	5.2 (45)	85	70 (18)	412	338 (18)	410	246 (40)	335	219	42	31	998	407	3.5	1.4	5.9	3.7
	L. H.	7.5	4.1 (45)	150	135 (10)	525	453 (14)	165	108 (35)	145	91	38	34	710	463	2.6	1.7	4.9	2.5

*In each case, the values listed under (C) represent the average of the last 3 control periods and those listed under (A) represent the average of the second, third, and fourth periods after standing. The numbers in parentheses refer to the per cent of fall from the control values in each case.

Key to abbreviations: UF = urine flow; GFR = glomerular filtration rate; RPF = renal plasma flow; Cosm = osmolal clearance; CH_2O = free water clearance.

UF, GRF, RPF, Cosm, and CH_2O are given in milliliters per minute. Na, Cl, and K are given in microequivalents per minute. Total solute is given in microosmoles per minute.

above 2.5 ml. per minute. After standing, the free water clearance fell considerably and during the last trimester of pregnancy it became negative throughout the last 2 to 3 experimental periods. The osmolal clearance (Cosm) also decreased but the fall was proportionately less than that of the free water clearance (Table I and Fig. 1).

Comparison of the changes in urine flow, glomerular filtration rate, renal plasma flow, and sodium excretion induced by standing in the 7 patients during the first, second, and third trimesters of pregnancy and in the postpartum period can be made from Table I. It is evident that in each subject the response to standing increased progressively with the progress of gestation and reached its maximum in the third trimester of pregnancy (Table I). In the first trimester, the free water clearance was never negative, whereas in the second and particularly in the third trimester it approached zero or became negative in all of the subjects (Table I). In the postpartum period the response was significantly less than that observed at any time during pregnancy. In fact, of the 4 patients tested in the postpartum period, 2 had a fall in urine flow and sodium excretion but had insignificant changes in the average glomerular filtration rate and renal plasma flow. Both of these patients had had a marked fall in glomerular filtration rate and renal plasma flow, particularly during the latter part of their gestation (Table I).

TABLE II. DATA ON UF, GFR, RPF, AND Na, Cl, K, AND SOLUTE EXCRETION OBTAINED FROM THE LIMB OCCLUSION EXPERIMENTS*

PATIENT	UF†		GFR†		RPF†		Na‡		Cl‡		K‡		TOTAL SOLUTE§	
	C	A	C	A	C	A	C	A	C	A	C	A	C	A
A. L.	21.3	2.3	150	85	700	395	225	33	193	50	47	23	987	379
K. J.	23.5	2.0	176	134	900	650	275	75	145	45	55	40	1,495	370
J. B.	16.3	1.4	148	101	950	480	142	45	110	20	80	48	750	527
A. An	7.8	0.9	134	90	650	455	138	80	98	20	44	40	741	322
T. A.	10.5	1.1	168	101	610	480	160	60	110	48	38	30	1,312	451

*In each case, the control values (C) represent the average of the last 3 control periods. The values listed under (A) represent the average of the second, third, and fourth periods after occlusion. All figures are corrected for body surface area.

†UF, GFR, and RPF are given in milliliters per minute.

‡Na, Cl, and K clearances are given in microequivalent per minute.

§Total solute is given in microosmoles per minute.

Calculation of the significance of the differences between the values of urine flow, glomerular filtration rate, renal plasma flow, and sodium excretion observed throughout the course of gestation and in the postpartum period was made by computing Student's "t" for each pair of values. The difference between third and first trimesters was highly significant ($P < 0.002$); the difference between postpartum and third trimester had a $P < 0.001$; the difference between second and first trimesters had a $P < 0.01$ and the difference between first trimester and postpartum had a $P < 0.05$.

Group II. Limb Occlusion.—The systemic effects of rapid venous occlusion of the lower extremities in the 5 pregnant patients studied were similar to but not as severe as those of quiet standing. In addition to the vasovagal symptoms, the patients had tingling of the toes and painful sensations in the feet.

Although the limb occlusion experiments were carried out on even a higher water diuresis than those of quiet standing, the renal changes were very similar. Table II presents the mean values for each patient before and after occlusion, whereas Fig. 2 illustrates a typical example of a complete experiment. Control urine flow varied from 7.8 to 23.5 ml. per minute. In the first period after cuff inflation, the urine flow fell moderately but continued to decline in subsequent periods until it reached values of 0.9 to 2.3 ml. per minute (Table II and Fig. 2).

Renal plasma flow and glomerular filtration rate likewise decreased progressively after cuff inflation and the magnitude of fall was approximately equal to that of quiet standing.

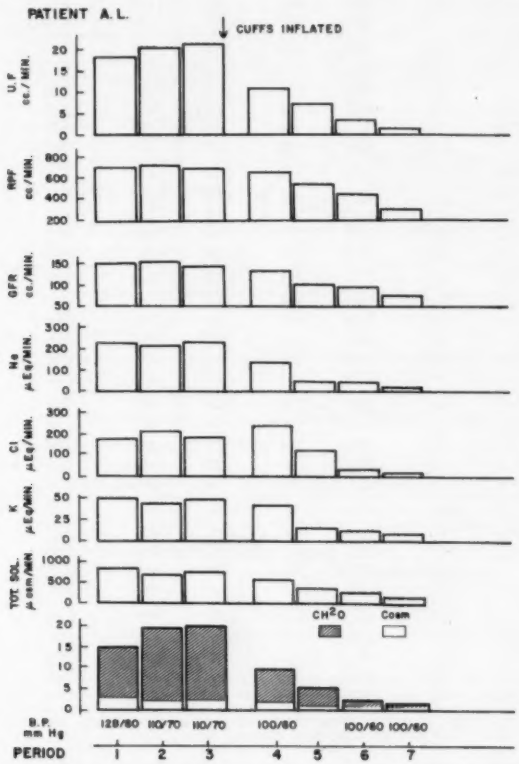

Fig. 2.—The effects of venous occlusion of the lower extremities on renal hemodynamics and water and electrolyte excretion in the last trimester of pregnancy. Note the similarity between the changes induced by venous occlusion and those of quiet standing illustrated in Fig. 1.

The changes in the excretion of sodium, chloride, potassium, and total solutes and in the free water and osmolal clearances were quite similar to those observed after quite standing (Table II and Fig. 2).

Group III. Alcohol Experiment.—All of the 7 patients included in this group had the systemic and cerebral manifestations which are usually observed after alcohol ingestion. Table III presents complete data on the renal effects of standing in the 5 patients who were given oral alcohol and in the 2 who

TABLE III. DATA ON UF, GFR, RPF, Na, Cl, K, AND SOLUTE EXCRETIONS, AND OSMOLAL AND FREE WATER CLEARANCES OBTAINED FROM PATIENTS WHO RECEIVED ORAL AND INTRAVENOUS ALCOHOL*

PATIENT	UF	GFR	RPF	Na	Cl	K	TOTAL SOLUTE	COSM	CH_2O
S. G. (Oral)	27.5	190	1,210	331	252	64	1,510	5.4	22.1
	10.0	155	1,100	298	95	58	1,130	4.0	6.0
	13.9	211	1,310	339	121	64	1,432	5.1	8.8
	1.9	95	761	107	25	49	538	1.9	0.0
	1.2	110	810	100	38	54	510	1.8	-0.6
P. O'C. (Oral)	21.7	158	722	264	202	36	1,440	5.2	16.5
	21.4	146	745	314	244	34	2,170	7.5	13.9
	3.7	135	698	235	123	32	1,215	4.2	-0.5
	4.8	130	693	74	70	43	1,245	4.3	0.5
	2.5	110	574	64	61	39	850	2.9	-0.4
	1.8	108	650	60	52	42	620	2.2	-0.4
M. W. (Oral)	28.2	170	869	200	183	68	1,252	4.5	23.7
	18.9	145	652	202	190	46	1,600	5.6	13.3
	0.9	92	610	30	20	14	407	1.4	-0.5
	1.2	122	772	21	18	38	452	1.6	-0.4
	1.6	140	794	65	48	107	814	2.9	-1.3
L. F. (Oral)	15.0	160	710	208	179	65	725	2.6	12.4
	11.2	150	590	112	130	50	637	2.3	8.9
	8.0	130	580	100	92	36	510	1.8	6.2
	0.7	88	440	18	11	20	270	1.0	-0.3
	0.9	56	240	29	23	24	280	1.0	-0.1
	1.1	85	421	53	32	47	345	1.2	-0.1
A. G. (Oral)	10.6	138	650	170	165	95	1,084	3.9	6.7
	7.0	99	388	86	57	80	719	2.5	4.5
	2.8	72	312	24	21	61	456	1.6	1.2
	1.4	70	303	16	12	49	323	1.2	0.2
	0.8	81	354	15	9	50	311	1.1	-0.3
B. B. (Intravenous)	10.8	133	810	155	135	85	790	2.8	8.0
	9.5	110	749	141	105	66	812	2.9	6.6
	1.1	113	527	100	91	68	596	2.1	-1.0
	1.1	129	532	85	80	66	617	2.2	-1.1
	1.3	120	529	111	82	86	680	2.4	-1.1
S. L. (Intravenous)	12.0	139	457	190	122	60	810	2.9	9.1
	3.2	110	410	127	100	54	477	1.7	1.5
	0.9	51	261	39	26	26	278	1.0	-0.1
	1.2	57	278	32	20	36	298	1.1	0.1
	1.6	87	390	60	38	55	410	1.5	0.1

*The figures above the line represent the average control periods and those below the line represent the successive clearances obtained during standing.

received this agent intravenously. Fig. 3 illustrates a complete experiment with oral alcohol. Control urine flow while the patient was recumbent varied between 10.6 and 28.2 ml. per minute (Table III). After standing and regardless of the route of alcohol administration, the urine flow fell considerably and the changes followed the same patterns observed after quiet standing without alcohol or after venous occlusion of the lower extremities. Similarly, the

changes in glomerular filtration rate, renal plasma flow, and the excretions of sodium, chloride, potassium, and total solutes were identical with those observed during quiet standing without alcohol (Table III and Fig. 3). Because of the high rate of water diuresis used in these patients, control free water clearance varied from 8 to 23 ml. per minute. After standing and despite the alcohol, it fell considerably and became negative in every case. Osmolal clearance also followed a pattern similar to that of quiet standing without alcohol.

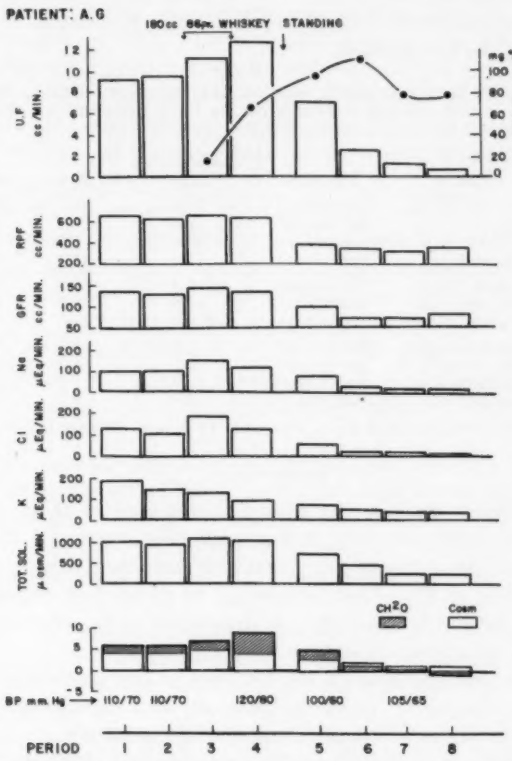

Fig. 3.—Effects of quiet standing in a subject to whom alcohol was given orally. A total of 180 ml. of 86 proof whiskey was ingested between the third and the fourth control periods while the patient was still recumbent. Blood levels of alcohol (●——●) were determined immediately after the beginning of ingestion and continued for the entire experiment. Note that despite the high blood level of alcohol, the changes in UF, GFR, and excretions of Na, Cl, K, and total solutes were similar to those of quiet standing without alcohol. Here again free water clearance became negative in the last 2 clearance periods.

Group IV. Aldosterone Excretion.—Table IV shows the output of aldosterone, sodium, potassium, and urine in the 3 patients during recumbency and ambulation.

The first patient excreted practically the same amount of aldosterone while she was recumbent in bed during the day and at night. During the 12

hours of ambulation, her aldosterone excretion rose markedly. On the following 12 hours, when she again reassumed the recumbent position in bed, her aldosterone output returned to control recumbent levels. Sodium excretion fell significantly during ambulation and rose during recumbency.

Potassium excretion was not altered significantly during the periods of ambulation and recumbency but urine flow fell during the period of ambulation (Table IV).

The other 2 patients had a similar striking increase in aldosterone excretion during ambulation, even though one of these patients ambulated for only 6 hours. Their patterns of excretion of sodium, potassium, and urine were similar to that of the first patient.

TABLE IV. EXCRETION OF ALDOSTERONE, SODIUM, AND POTASSIUM DURING RECUMBENCY AND AMBULATION BY 3 PREGNANT PATIENTS IN THE LAST TRIMESTER OF GESTATION*

PATIENT	TIME	POSITION	ALDOSTERONE (μg)	Na (mEq.)	K (mEq.)	URINE FLOW (ml.)
1	7 A.M. TO 7 P.M.	Recumbent	11.5	14.4	30.4	1,400
	7 P.M. TO 7 A.M.	Recumbent	12.6	4.4	25.0	1,360
	7 A.M. TO 7 P.M.	Ambulatory	45.0	0.9	20.3	930
	7 P.M. TO 7 A.M.	Recumbent	10.0	16.0	28.3	1.600
2	7 P.M. TO 7 A.M.	Recumbent	8.0	26.6	14.0	1,500
	7 A.M. TO 7 P.M.	Ambulatory	48.0	6.0	18.5	530
	7 P.M. TO 7 A.M.	Recumbent	6.0	14.5	16.8	950
3	7 P.M. TO 1 A.M.	Recumbent	4.5	12.5	18.0	400
	1 A.M. TO 7 A.M.	Recumbent	3.0	4.0	10.6	310
	7 A.M. TO 1 P.M.	Ambulatory	22.0	1.2	22.5	295
	1 P.M. TO 7 P.M.	Recumbent	3.8	8.7	20 0	860

*The values for Patients 1 and 2 are given per 12 hours, whereas those of Patient 3 are per 6 hours.

DISCUSSION

Despite extensive studies, the stimuli which lead to the antidiuresis and antinatriuresis of quiet standing still remain controversial. According to a recent review of this subject[20] the available evidence, which is largely inferred from studies on the effects of alcohol on urine flow, tends to favor the hypothesis that the fall in urine flow is dependent to a large extent on excessive liberation of antidiuretic hormone.

However, the contribution of the changes in the glomerular filtration to either the antinatriuresis or to the antidiuresis still remains a disputed question. Epstein and his co-workers[4] suggested the existence of separate mechanisms for the antidiuresis and the antinatriuresis and attributed the latter to the concomitant fall in filtration rate and, therefore, the reduced filtered load. Pearce and Newman,[2] although accepting the dual mechanisms, found the changes in the endogenous creatinine clearance in moderately hydrated subjects too small to account for the marked fall in electrolyte excretion. Surtshin and White[6] reached essentially the same conclusion on the basis of studies carried out on well-hydrated patients.

Our data obtained throughout the course of normal gestation show that the antinatriuresis of quiet standing or of venous occlusion of the lower extremities is closely associated with a fall in glomerular filtration rate. These

findings are in agreement with those of Epstein[4] and suggest that the changes in glomerular filtration may play an important, if not a dominant, role in the antinatriuresis of quiet standing in pregnant women.

Our failure to inhibit the antidiuresis of quiet standing with alcohol in pregnant women is contrary to the observations of others[1-3] in nonpregnant subjects, although the method of administering alcohol and the dose used were practically the same. Two possible reasons can be offered to explain this divergency. First, that in pregnancy the stimuli which provoke the elaboration of ADH during orthostasis are so strong and the quantity of hormone liberated so high that a mild inhibitor such as alcohol could not block the marked antidiuresis. Testing of this hypothesis will have to await the development of more accurate methods for ADH determinations in body fluids. The second possibility is that the marked fall in glomerular filtration rate which occurs in pregnant women during standing may account totally or partially for the decrease in urine flow.

This latter hypothesis is favored by available evidence. Judson and his associates[21] observed that patients with diabetes insipidus who had a negligible decrease in blood pressure after venous congestion of the lower limbs had only a slight fall in urine flow with the usual fall in glomerular filtration rate and sodium excretion. In contrast, the patients with diabetes insipidus who developed vasovagal collapse had a marked antidiuresis along with the antinatriuresis and the fall in renal plasma flow and glomerular filtration rate. Such changes persisted even after the blood pressure had returned to control values.

These latter findings are very similar to those observed in pregnant patients since practically all of them developed clinical manifestations of vasovagal collapse during standing or venous congestion. In fact, several patients developed vasovagal symptoms while lying supine. Therefore, it may be presumed that the mechanism of their oliguria might have been similar to that of the patients with diabetes insipidus reported by Judson and his co-workers. Although these authors at that time thought the antidiuresis to be due to "certain intrinsic renal adjustments," in the light of recent observations[22, 23] it could be attributed to the fall in glomerular filtration rate. Similar fall in urine flow in the presence of a reduced filtration rate has been recently described by del Greco and de Wardener,[22] Berliner and Davidson,[23] and Kleeman and associates.[24]

These investigators have shown that reduction of glomerular filtration rate in dog or man results in a marked fall in urine flow and the elaboration of a concentrated urine independently of ADH. Berliner[23] found that the oliguria was the result of the reduction in the free water clearance which fell more markedly than the osmolal clearance. Such behavior in the 2 moieties which compose the urine flow occurred in the pregnant subjects presented in this report.

Our values for aldosterone excretion during the control periods in recumbency were high but consistent with values of normal pregnancy reported

by others.[25] However, the data failed to show any diurnal variation. The striking rise which occurred when the patients were standing and ambulating and the fall observed when the same patients reassumed the recumbent position indicates that the rise was strongly related to the upright position rather than to true diurnal variation. However, it should be realized that the studies on aldosterone excretion have been made during chronic ambulation, whereas the renal factors have been assessed in acute states of quiet standing. Therefore, the homeostatic mechanisms operating in these two situations may be different. Although this possibility cannot be ruled out, our results during 6 hours of ambulation and those of Gowenlock and his co-workers[26] obtained from pooled urine collected during 2 hours of standing indicate that aldosterone plays some role in the orthostatic antinatriuresis. This opinion is shared by Muller and his associates[8] and by Bartter and his co-workers.[12] In the light of the present data, it is interesting to attempt to reconstruct the possible chain of events which lead to the antidiuresis and antinatriuresis of quiet standing in pregnancy. Quiet standing or venous congestion of the limbs will produce marked venous pooling in the lower extremities which will decrease the effective circulating blood volume and the cardiac output. This would lead to (1) a decrease in the renal plasma flow and glomerular filtration rate, (2) an increase in aldosterone secretion by the adrenal cortex, and (3) the possible liberation of ADH by the neurohypophysis. It is probable that the role that each plays in the antidiuresis and antinatriuresis depends on a multiplicity of factors and particularly on the degree of blood pooling. In pregnant subjects, because of the greater degree of pooling, the primary factor responsible for the antidiuresis and antinatriuresis is the change in renal hemodynamics. Aldosterone may become an important factor even when the glomerular filtration rate has returned to prestanding values. It is difficult, however, to state from the present data when aldosterone action begins and for how long it may continue. It is also difficult to exclude the possibility that ADH might still be operating to a certain extent in pregnancy even though glomerular filtration rate and aldosterone seem to be the predominant factors.

That the renal hemodynamic changes induced by orthostasis in pregnancy are more striking than those of nonpregnant subjects, is evident from our data which show clearly that in every instance the postpartum response was much less than that observed during pregnancy. Among the reasons which could account for this fact are the increased venous pressure in the lower extremities, the increased intra-abdominal pressure and the pressure of the heavy uterus lying over the major vessels, and the possible existence of an arteriovenous shunt at the level of the placenta. These factors would favor a greater shift in blood volume and the trapping in the lower extremities of a larger portion of the effective circulating blood volume upon standing or venous occlusion. This hypothesis receives support from the well-known fact that pregnant women faint easily when they stand for some time or even when they lie supine on a hard surface.[14] It is also favored by the appearance of edema in

normally pregnant subjects upon standing, particularly after the sixth month of gestation. It is possible that this type of edema is promoted by the standing position through the mechanisms already discussed. Its disappearance when the patient remains at bed rest or during the night favors the hypothesis that the standing position is closely connected with its etiology.

SUMMARY AND CONCLUSIONS

1. Studies on the effects of blood pooling on renal hemodynamics and water, electrolyte, and aldosterone excretion were made in 22 normally pregnant patients during moderate and severe water diuresis.

2. The changes induced by quiet standing were followed in the same patient throughout the course of gestation and through the postpartum period. The results showed a marked decrease in water, sodium, and chloride excretion which was concomitant with a significant but less marked fall in glomerular filtration rate and renal plasma flow. The excretion of potassium was irregular but in general it also decreased. These effects were much more striking during pregnancy than in the postpartum period.

3. Venous congestion of the lower extremities produced renal and systemic effects closely similar to those of quiet standing.

4. Oral and intravenous administration of alcohol did not inhibit the antidiuresis or the antinatriuresis of quiet standing and did not alter the fall in glomerular filtration rate and renal plasma flow.

5. Aldosterone excretion did not show diurnal variation but rose markedly during standing and ambulation.

6. It is suggested that in pregnant women, because of the more marked pooling in the lower extremities, the fall in glomerular filtration rate may account for the initial antidiuresis and antinatriuresis of quiet standing. Aldosterone and possibly ADH may enter into play later and maintain these changes.

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COMPARISON OF THE SENSITIVITY OF SEVERAL MAMMALIAN CELL TYPES FOR THE ISOLATION OF POLIOMYELITIS VIRUSES FROM MAN

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MONOLAYER cell cultures are now widely used for the isolation of certain viruses from clinical materials. A large variety of cell types—malignant, embryonic, and adult—derived from a variety of tissues from several animal species is currently available, but the range of viruses to which all of these cell types are susceptible is still unknown in large part. In the present study, 4 commonly used cell types were compared for their sensitivity in the detection of poliomyelitis virus in human fecal specimens. Because of their possible bearing on clinical and epidemiologic studies, the comparisons were made under conditions which might prevail in any laboratory attempting isolations of poliomyelitis virus on a fairly large scale. The cell types compared were the normal human amnion and normal monkey kidney cells, and strain HeLa and strain KB cells derived from human carcinomas.

MATERIALS AND METHODS

Tissue Culture Procedures.—The various cell lines were utilized in the form of monolayer cell cultures in tubes.

Monolayer cultures of monkey kidney cells were prepared essentially according to the method outlined by Youngner.[1]

Strain HeLa cells (Gey) were maintained in continuous culture by the method described elsewhere.[2] Monolayer cell cultures in tubes were prepared according to the procedure given in an earlier publication.[2]

Strain KB cells[3] were maintained in continuous passage by the same procedure employed for strain HeLa cells. Monolayer cell cultures in tubes were prepared by the same method as that used for strain HeLa cells.

Human amnion cells were prepared by a modification of the general method outlined by Zitcer and associates.[4]

Stool Suspensions.—Stools were made into approximately 20 per cent suspensions by shaking with Hanks' balanced salt solution in a 50 ml. stoppered centrifuge tube containing glass beads. The suspension obtained after vigorous manual shaking was centrifuged horizontally for 10 minutes at 3,000 r.p.m. The supernatant fluid was decanted through sterile gauze

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into a suitable glass vessel and thence decanted into Lusteroid tubes, and centrifuged for 1 hour at 10,000 r.p.m. in a Spinco Model L centrifuge. The supernatant fluid was distributed into two tubes which were stoppered, labeled, and stored at −20° C. until used for inoculation.

All stool suspensions employed in this study were previously unthawed aliquots of suspensions which had been inoculated into monkey kidney cell cultures during the course of other studies. The results of these tests in monkey kidney cells were not known to the individuals carrying out the present study. The stool suspensions studied were selected by another investigational group, and were selected to provide a series of specimens the majority of which were known to contain poliomyelitis virus. Because of the geographical origin of the specimens (California, Arizona, and Nevada), most of the virus-containing stools contained type 1 poliomyelitis virus and a small proportion contained the type 3 virus; type 2 poliomyelitis virus was not represented by any of the stools chosen for the study. Since the study was primarily to determine the sensitivity of the various cell types to poliomyelitis viruses, stools known to contain viruses other than poliomyelitis were not intentionally included in the inquiry.

Inoculation of Cultures With Stool Suspensions.—Prior to inoculation, the cell sheets in the culture tubes were rinsed 3 times using 1 ml. of Hanks' BSS for each rinse. Maintenance medium, consisting of equal parts of medium 199 and Hanks' BSS with 5 per cent horse serum (inactivated at 56° C. for 30 minutes) was then added to each tube in a volume of 0.9 ml. Stool suspensions were thawed, thoroughly mixed by pipetting, and 0.1 ml. of suspension was added to the culture tube. Each suspension was tested in 4 tubes of each tissue type, and 4 uninoculated control tubes were included with every group of 80 or fewer tubes of each tissue type.

Interpretation of Results.—After inoculation, all tubes were incubated in stationary racks at 36° C., and were examined daily as long as the cell sheet remained sufficiently healthy to permit detection of viral cytopathogenesis. Where cytopathogenesis occurred, it was allowed to progress until approximately 75 per cent of the cells in each tube of a set of 4 was affected, at which time the fluids from the 4 tubes of the set were pooled and stored at −20° C. Where only one or two tubes of the 4 showed cytopathogenesis, cellular degeneration was permitted to proceed until all cells in the affected tubes were involved before the fluids of the 4 tubes were pooled. Where cytopathogenesis was suspected to be due to toxic or nonspecific factors, and was so marked as to make the tubes unreadable in less than 7 days after inoculation, the contents of the tubes were pooled for an additional passage. Tubes showing no toxic or viral cytopathic effects were held as long as they remained readable, and were then discarded. The criterion of readability employed was the persistence of at least two low-powered fields of continuous cell sheet free of changes that could obscure detection of a viral cytopathogenic effect.

Tests were done in groups of 19 stool suspensions so that each test consisted of 320 culture tubes, including the controls. All the tubes of one test were examined by a single observer, who usually read the entire series of tubes at one sitting. Consequently, the individual making the readings was sometimes aware of the effect of a given stool suspension on one of the cell types while interpreting the effect of the same suspension in the remaining cell types. This procedure represents a possible source of bias in interpretation and could not be avoided entirely; on the other hand, it avoids the subjective differences inherent in 4 sets of readings made by 4 different observers.

The results were recorded as follows:

Positive: The appearance, at any time, or viral cytopathogenic effect in at least one of a set of 4 tubes.

Negative: Persistence of a healthy cell sheet in the inoculated tube for a length of time equal to the period of survival of uninoculated cell sheets (controls) and for at least 7 days.

Toxic: Degeneration of cell sheets in less than 7 days in a manner not typical of viral cytopathogenesis, the control remaining readable during the same period.

Doubtful: Degeneration of cells in a manner suggestive, but not typical, of viral cytopathogenesis. (Most interpretations of "doubtful" represented mixtures of toxic and viral cytopathic effects.)

Subcultures.—Subcultures were made from all sets of tubes in which the cytopathic effects were interpreted as positive, toxic, or doubtful. No further studies were made on sets of cultures considered negative.

All second passages were made in the same cell type as that used for the initial passage, and the tubes were examined, read, and handled in the same manner as the first passage tubes. Whenever results of the second passage were interpreted as toxic or doubtful, a third passage was made.

For the second and third passages, 0.1 ml. of pooled culture fluids was used as inoculum.

Typing of Virus Isolates.—In the original test using monkey kidney cell cultures as the host system, all the viruses recovered were identified as poliomyelitis viruses. Consequently, in view of the fact that 4 different cell types were being employed in the comparative studies, it appeared desirable to identify each virus isolate to determine whether the agents recovered in each cell type were actually poliomyelitis viruses or some other virus. Since a total of 255 isolates was involved, virus typing was performed by a modification of the colorimetric test for poliomyelitis virus,[5-7] the test used being similar to that devised in this laboratory for the typing of adenoviruses,[8] Coxsackie viruses,[9] and Western equine encephalomyelitis virus.[10]

Strain HeLa and human amnion cells were used in the colorimetric tests, and the special metabolism medium employed differed according to the cell type used. For strain HeLa cells the following medium was used.

Horse serum (inactivated 56° C. for 30 minutes), 5.0 per cent.

20 per cent glucose, 1.5 per cent.

Lactalbumin hydrolyzate-yeast extract medium, 93.5 per cent.

8.8 per cent $NaHCO_3$, 1.25 ml. per 100 ml. medium.

Antibiotics: penicillin 125 units per milliliter; streptomycin and neomycin 125 μg of each per milliliter; bacitracin 1.25 μg per milliliter; and mycostatin 100 units per milliliter.

The metabolism medium used with human amnion cell systems was similar to the above, the difference lying in the omission of the 20 per cent glucose and the replacement of lactalbumin hydrolyzate-yeast extract medium with a mixture of equal parts Hanks' BSS and lactalbumin hydrolyzate-yeast extract medium, i.e., 47.5 per cent of each.

The tests were done in polystyrene panels after the method described by Melnick and Opton.[11] The cells, the virus isolates, and the type-specific antisera were added to the cups in 0.2 ml. amounts. The cups were sealed by adding 0.7 ml. of a sterile light mineral oil. Human amnion cells were used in concentrations of 120,000 cells per 0.2 ml. and strain HeLa cells were used in concentrations of 50,000 cells per 0.2 ml. The virus isolates were diluted 1×10^{-2} in the special metabolism media. Type-specific antisera (monkey) to the 3 immunologic types of poliomyelitis virus were inactivated at 56° C. for 30 minutes and diluted in metabolism medium in accordance with the known neutralizing capacity of the serum. Appropriate controls (cell titrations, viral activity) were included in each test.

The mixtures were incubated at 35° C. until the cell controls included in the test had reached a pH of 7.0 or less. In general, viral activity was indicated by a final pH of 7.8 to 8.0 and neutralization by a pH of 6.6 to 7.0. In a number of instances where the viral isolates had a low titer, these differences were not clearly defined and the test was repeated using a higher concentration of virus.

Isolation of Viruses.—For purposes of this study, the enteric virus unit of this laboratory supplied 85 stool specimens which had previously been tested in monkey

kidney cell cultures. Table I gives the results of the original tests on these stool specimens and is presented in order to facilitate comparisons with data derived from the comparative tests of the several cell types employed in the present inquiry. Thus, it will be noted that only the type 1 and type 3 poliomyelitis viruses were represented, and that 70 (82 per cent) of the specimens yielded a poliomyelitis virus and that 15 specimens (18 per cent) yielded no virus.

TABLE I. RESULTS OF ORIGINAL VIRUS ISOLATION ATTEMPTS IN MONKEY KIDNEY CELL CULTURES OF STOOL SUSPENSIONS RETESTED IN COMPARATIVE STUDY*

RESULTS OBTAINED IN ORIGINAL STUDY		
	VIRAL ISOLATIONS	
VIRUS SEROTYPE	NUMBER	PER CENT
Type 1	58	68.3
Type 3	12	14.1
Total positive	70	82.4
	No isolation	
—	15	17.6
Total stools tested	85	100.0

*See Materials and Methods.

TABLE II. INTERPRETATION OF RESULTS OF PRIMARY PASSAGE OF 85 STOOL SUSPENSIONS IN MONOLAYER CULTURES OF 4 DIFFERENT CELL TYPES

	INDICATED EFFECTS OBSERVED IN CULTURES OF							
	HUMAN AMNION		MONKEY KIDNEY		HeLa CELLS		KB CELLS	
EFFECT OF INOCULUM (CPE*)	NO. SUSPENSIONS	PER CENT	NO. SUSPENSIONS	PER CENT	NO. SUSPENSIONS	PER CENT	NO. SUSPENSIONS	PER CENT
Positive	52	61.2	53	62.4	48	56.4	52	61.2
Negative	16	18.8	18	21.2	16	18.8	16	18.8
Doubtful	3	3.5	3	3.5	6	7.1	2	2.4
Toxic	14	16.5	11	12.9	15	17.6	15	17.6

*CPE = cytopathogenic effect.

RESULTS

As just mentioned, and as shown in Table I, a poliomyelitis virus was isolated from 70 of the 85 stools on original test in monkey kidney cell cultures. It will be noted that the results obtained in the present study are not strictly comparable to those obtained in the original or initial tests since the clear fluids representing processed stool suspensions had been stored for some time and different methods of inoculation were used. For evaluation of the sensitivities of the various cell types to poliomyelitis viruses, comparisons should be made between the results obtained in cultures of the several different cell types which were, in essence, inoculated simultaneously with the same inoculum by the same procedure.

The results obtained on initial culture of the 85 stool suspensions are summarized in Table II. The differences in the virus recovery rates in the 4 cell types are small. The greatest number of isolations was made in monkey kidney cells and the lowest number in strain HeLa cells, but the proportional difference between the two recovery rates, namely, 62 per cent versus 56 per cent, was not found to be statistically significant when the chi-square test was applied. The difference between monkey kidney cell cultures and strain

HeLa cell cultures with respect to the number and proportion of specimens which gave rise to "doubtful" or "toxic" interpretations at the first passage level is somewhat more striking; thus the number of instances in which these interpretations were made was 14 (16.4 per cent) and 21 (24.7 per cent) for monkey kidney cell and strain HeLa cell cultures, respectively. Evidence that this difference is statistically significant is lacking ($p=0.18$) and this suggestion that strain HeLa cells are more susceptible to toxic factors than are monkey kidney cells can only be interpreted as in agreement with the impression of other workers on this subject. Human amnion and strain KB cells appeared (Table II) to occupy a position intermediate between monkey kidney and strain HeLa cells with respect to susceptibility to toxic factors.

TABLE III. FINAL RESULTS, IN TERMS OF VIRUS ISOLATIONS, OBTAINED WITH 85 STOOL SUSPENSIONS IN MONOLAYER CULTURES OF 4 DIFFERENT CELL TYPES*

VIRUS ISOLATION ATTEMPT	RESULTS OBTAINED IN CULTURES OF							
	HUMAN AMNION		MONKEY KIDNEY		HeLa CELLS		KB CELLS	
	NO. SUSPENSIONS	PER CENT	NO. SUSPENSIONS	PER CENT	NO. SUSPENSIONS	PER CENT	NO. SUSPENSIONS	PER CENT
Positive	64	75.3	63	74.1	63	74.1	65	76.5
Negative	21	24.7	22	25.9	22	25.9	20	23.5

*Results based on cytopathogenic effect observed at second or third subpassage.

Cultures which showed cytopathic effects that were interpreted as doubtful or toxic were subcultured until a clear-cut positive or negative result was obtained. Three such subcultures were required for only 3 specimens, all the remainder requiring no more than two. The final recovery rates in the 4 types of cell cultures are shown in Table III. Comparison of Tables II and III will show that clear-cut evidence of specific cytopathic effects was obtained at the first passage level in a majority of instances, and that additional virus recoveries were made when "doubtful" or "toxic" specimens were subpassaged; the number of additional recoveries made on subculture is readily discernible. Table III shows that the final results in terms of virus recovery were practically identical for the 4 cell types. The greatest difference encountered, and of no practical or statistical significance, was between strain KB cells in which 65 of the 85 stool specimens tested yielded a virus, and monkey kidney and strain HeLa cells in which 63 of the 85 specimens yielded a virus.

Although the virus recovery rates in the 4 different cell types were essentially identical, there were some interesting inconsistencies in virus recovery in several instances. Seven stool suspensions were involved. Of these, 5 gave specific cytopathic effects in 3 of the 4 cell types. One specimen was positive in 2 cell types and negative in 2 others. One specimen affected a single cell type only. Of the 7 stool specimens involved, 5 produced cytopathic effects in only one or two tubes of the sets of 4 which gave the positive result.

Table III shows that a total of 255 agents producing cytopathic effects in the tissue culture systems used was recovered. Since cell types other than

monkey kidney were being utilized, it appeared necessary to determine whether the agents recovered in these various cell culture systems were identical with those originally isolated from the specimens in monkey kidney cell cultures. Consequently, all 255 isolates were tested by the colorimetric neutralization test described above, using monkey hyperimmune sera to each of the 3 immunologic types of poliomyelitis virus. Of these isolates, 232 (91 per cent) were successfully typed on the first attempt, and in each instance the virus type recovered agreed with that previously recovered in the initial cultures in monkey kidney cell cultures. The remaining 23 isolates were retested and 16 were successfully typed in this repeat test. The remaining 7 isolates were tested a third time; 3 were typed successfully on this occasion and 4 were subsequently found to be mixtures of poliovirus and an unidentified agent. In each of these 23 cases, the virus type recovered was the same as that isolated in the original cultures conducted in monkey kidney cell systems.

TABLE IV. PROPORTION OF VIRUS ISOLATES WITH TITER OF 1×10^2 OR MORE TISSUE CULTURE DOSES

PASSAGE LEVEL	VIRUS ISOLATED IN CULTURES OF			
	HeLa CELLS	MONKEY KIDNEY CELLS	KB CELLS	HUMAN AMNION CELLS
First	31/31	44/47	27/30	39/49
	100 %	94 %	90 %	80 %
Second	30/30	16/16	32/32	12/12
	100 %	100 %	100 %	100 %

On the whole, inability to type the isolate colorimetrically was due to the low titer of the isolate, i.e., less than 1×10^2 tissue culture doses, as indicated by the failure of the virus alone to kill the cells in the neutralization scheme used. This was true of 16 of the 23 isolates which could not be typed at the first attempt. In the remaining 7 instances, inability to type 3 of the isolates was referable to "break-through" due to the high titer of the isolate and in 4 instances was referable to break-through produced by a mixture of poliomyelitis virus and an unidentified agent.

While the evidence derived from this study indicates that the 4 cell systems utilized are of essentially equal sensitivity in detecting the presence of poliomyelitis viruses in clinical material, the results of some of the colorimetric typing tests suggest that the virus multiplies to a greater extent in some of the cell culture systems than in others. Evidence to this effect is presented in Table IV. Table IV shows that in the case of isolates obtained in strain HeLa cell cultures, 31 attempts were made to type the isolates from first passage culture fluids, and all 31 attempts were successful, i.e., the titer of the isolate was at least 1×10^2 tissue culture doses. Similarly, 27 of 30 isolates at the first passage level in KB cell cultures, 44 of 47 isolates at the first passage level in monkey kidney cell cultures and 39 of 49 isolates at the first passage level in human amnion cell cultures were typed successfully. On a proportional basis 100 per cent of the isolates from HeLa cell cultures had a sufficiently high titer to permit typing at the first passage level, whereas

only 80 per cent of the isolates from human amnion cell cultures had a sufficiently high titer to permit successful typing at this level. By the second passage level, however, the infectivity titers in all of the 4 cell culture systems were sufficiently high to permit of typing.

DISCUSSION

The findings presented in this communication indicate that for purposes of virus recovery from clinical materials, monkey kidney cells, strain HeLa cells, strain KB cells, and human amnion cells are equally sensitive to infection with the poliomyelitis viruses. Comparative titrations of stock viruses were not done as a part of this study since it was considered that any differences which might be encountered might reflect differences in adaptation to growth in a tissue culture system. It was desired rather to determine whether any one of the 4 cell systems examined possessed any advantages or superiorities in the detection of poliomyelitis viruses in clinical materials, and it would seem that from the standpoint of sensitivity at least, no one system is superior to any of the others. All of the cell types employed develop clear-cut cytopathogenic changes when infected with poliomyelitis viruses. The relative ease with which specific cellular damage is detected is often debated but is probably more a function of individual experience than of objective differences. Monolayer cultures of monkey kidney cells appeared to possess a slightly greater resistance to the toxic effect of some stool suspensions than did the other cell types tested, especially strain HeLa cells. On the basis of the 85 stool suspensions tested, the difference was not statistically significant. The toxic effect of certain stool suspensions, plus the occurrence of questionable cytopathogenic changes, will necessitate second passages in a variable proportion of instances.

SUMMARY AND CONCLUSION

An inquiry was made into the comparative sensitivity of monkey kidney, strain HeLa, strain KB, and human amnion cells in the detection of poliomyelitis viruses in stool suspensions. Essentially no difference was found in the efficiency of these 4 cell systems in the recovery of these viruses. The clinical specimens tested contained only type 1 or type 3 viruses.

Monkey kidney cells appeared to be somewhat more resistant to the effects of toxic factors in stool suspensions than were the other cell systems employed, but the difference was not found to be statistically significant.

The choice of the cell system to be employed for isolation of poliomyelitis viruses will depend on practical local considerations.

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A COMPARATIVE STUDY OF FETAL AND MATERNAL SERUM ENZYME LEVELS

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IN A previous study we presented the results of aldolase determinations in the newborn cord blood serum and compared them to the levels of the maternal blood serum at the time of delivery. It was found that the fetal aldolase activity was significantly higher than that of the mother and this activity increased during the first 4 days of the newborn. It was pointed out that certain conditions of intrauterine and neonatal life might be responsible for the differences between fetal and maternal levels of aldolase activity.[1] These differences might include such factors as increased adrenal activity, variations in muscular activity, and other factors of homeostasis.

This study of the enzymes in the mother at term and in the newborn at delivery was extended to include a group of esterases (cholinesterase, tributyrinase, and alkaline phosphatase) as well as glutamic oxalacetic transaminase and lactic dehydrogenase. The two latter enzymes, in addition to aldolase, are known to be increased in the serum of patients with myocardial infarction and liver and muscle necroses.

A knowledge of enzyme distribution in mother, fetus, and placenta may give some indication as to the site of enzymes synthesis and the function of these enzymes during pregnancy and neonatal life. Since enzymes are known to be large protein moieties, they would not be expected to diffuse across the placental barrier. The significant differences between enzyme activities in maternal and fetal serum found in our present study bear out this premise.

MATERIALS AND METHODS

Specimens of cord blood were collected immediately after delivery without attempt to differentiate arterial from venous flow. Venous blood was withdrawn from the mother within several minutes after delivery. The sera were separated, immediately refrigerated, and determinations performed as soon as convenient, mostly within 48 hours after collection.

The method for aldolase was described previously.[1] Glutamic oxalacetic transaminase was determined spectrophotometrically by the method of Karmen.[2] Lactic dehydrogenase was determined by the method of Wroblewski and LaDue[3] with pyruvic acid substrate and

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the spectrophotometric measurement of the disappearance of diphosphopyridine nucleotide (DPNH). Cholinesterase was measured by a colorimetric procedure introduced by Molander and associates[4] which determines the change in pH of a buffered acetylcholine substrate with phenol red as indicator. Tributyrinase was determined electrometrically by the method of Friedman and Shriro[5] and the results reported in pH changes of a buffered glycerol butyrate substrate. Alkaline phosphatase was determined by the method of Shinawara and co-workers[6] using glycerophosphate substrate buffered to pH 10.8. The transaminase and lactic dehydrogenase determinations were either carried out at 25° C. or corrected to that temperature by a correction factor of Q_{10} of 1.8 over a range of 23° to 33° C.[7]

TABLE I. CHOLINESTERASE IN MOTHER AND CORD SERA RECORDED IN COLORIMETRIC ΔpH UNITS

	MOTHER	CORD	t
Subjects	162	162	
Total range	0.34-0.86	0.36-0.77	
Average	0.605	0.576	2.88
S.D.	±0.092	±0.095	

TABLE II. TRIBUTYRINASE IN MOTHER AND CORD SERA RECORDED IN ΔpH UNITS

	MOTHER	CORD	t
Subjects	168	153	
Total range	0.40-0.87	0.36-0.82	
Average	0.607	0.566	3.19
S.D.	±0.115	±0.115	

RESULTS

The enzymes studied may be divided into the esterases (cholinesterase, tributyrinase, and alkaline phosphatase) and enzymes that are altered during necrotic processes (aldolase, transaminase, and lactic dehydrogenase). In the esterase group the levels in the sera of mothers at delivery were significantly higher than in the cord. The reverse was observed with the other enzymes, where markedly higher levels were found in the cord sera.

Table I gives a summary of cholinesterase values in mother and cord serum. The average was 0.605 △pH unit for the mother and 0.576 unit for the cord serum. Although relatively close, these differences were statistically valid with a t of 2.88 and $P < 0.01$.[8]

Table II shows results for tributyrinase similar to those for cholinesterase. In the mother the average was 0.607 and in cord serum the △pH averaged 0.566. The t was calculated at 3.19 and here also we have two statistically different groups with $P < 0.01$. There is some question whether one may be measuring the same enzyme even though different substrates are used, acetylcholine for cholinesterase and tributyrin for tributyrinase. Bunch and Emerson[9] report a coefficient of correlation of 0.905 for both enzymes. This study with a large number of subjects suggests that the same enzyme may be involved. The fact that the △pH values are numerically similar for both enzymes is fortuitous, but the relative activities in mother and cord and the similar t value suggest an identity of action. This similarity was observed in individual patients as well as in the average.

The values for alkaline phosphatase (Table III) show the maternal sera to be of different order of magnitude from the cord sera, with a t of 16.9. The average value of 11.9 units in the sera of mothers at delivery compares with the authors' range[6] of 2.2 to 8.6 for adults and to an average of 3.43 ± 0.81 in our laboratory for women in early pregnancy.[10] The average in the cord sera was 7.0 units, significantly lower than in the mother at the time of delivery.

The inorganic phosphate values, in Table IV, are an incidental finding as by-product of the alkaline phosphatase procedure which requires inorganic phosphate analysis. It is of interest, however, that the values for inorganic phosphate are significantly higher in the cord sera while the alkaline phosphatase is lower.

TABLE III. ALKALINE PHOSPHATASE IN MOTHER AND CORD SERA (RECORDED IN S.J.R. UNITS)

	MOTHER	CORD	t
Subjects	149	149	
Total range	5.5-19.1	3.8-11.3	
Average	11.9	7.0	16.9
S.D.	±3.4	±1.6	

TABLE IV. INORGANIC PHOSPHATE IN MOTHER AND CORD SERA (RECORDED IN MG. P PER 100 ML. SERA)

	MOTHER	CORD	t
Subjects	134	129	
Total range	2.1-6.2	3.7-7.6	
Average	3.9	5.4	16.1
S.D.	±0.73	±0.80	

TABLE V. GLUTAMIC OXALACETIC TRANSAMINASE IN MOTHER AND CORD SERA (RECORDED IN SPECTROPHOTOMETRIC UNITS)

	MOTHER	CORD	t
Subjects	161	157	
Total range	11-44	13-50	
Average	22.0	27.3	5.28
S.D.	±6.7	±8.2	

TABLE VI. LACTIC DEHYDROGENASE IN MOTHER AND CORD SERA (RECORDED IN SPECTROPHOTOMETRIC UNITS)

	MOTHER	CORD	t
Subjects	105	101	
Total range	80-362	135-520	
Average	177	300	8.15
S.D.	±67	±109	

In contrast to the esterases, the other enzymes investigated were higher in the cord sera. The aldolase figures were already reported.[1] Glutamic oxalacetic transaminase (Table V) shows an average value for the mother at 22.0 units and for the cord at 27.3 and with t equal to 5.28; the results are statistically valid with $P<0.01$. Similar results are seen in Table VI for lactic dehydrogenase where the mothers' levels average 177 units and the cord 300 units with a t of 8.15.

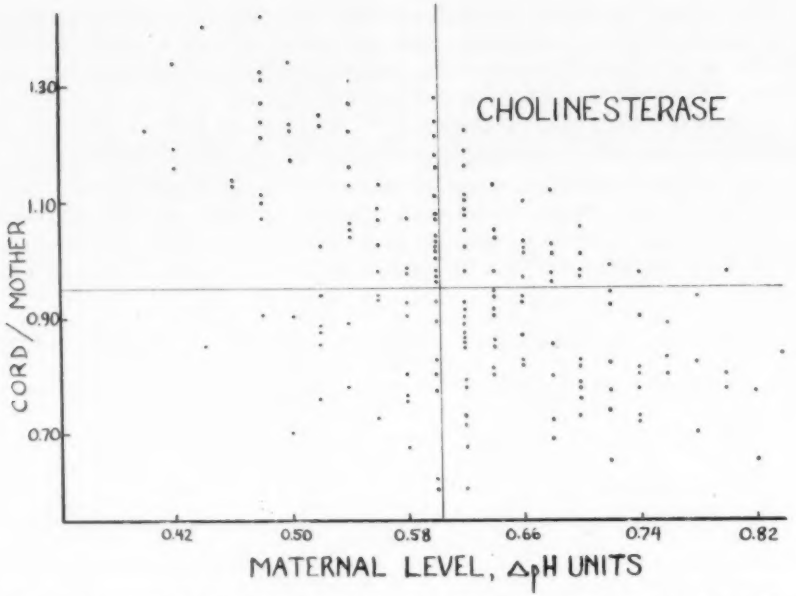

Fig. 1.—Scattergram of mother enzyme level and the corresponding cord/mother ratio for cholinesterase (see Table I).

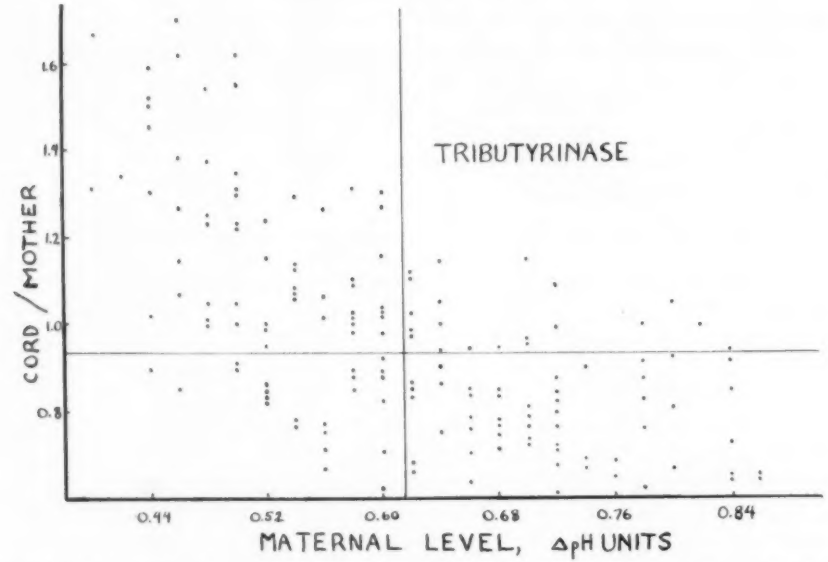

Fig. 2.—Scattergram of mother enzyme level and the corresponding cord/mother ratio for tributyrinase (see Table II).

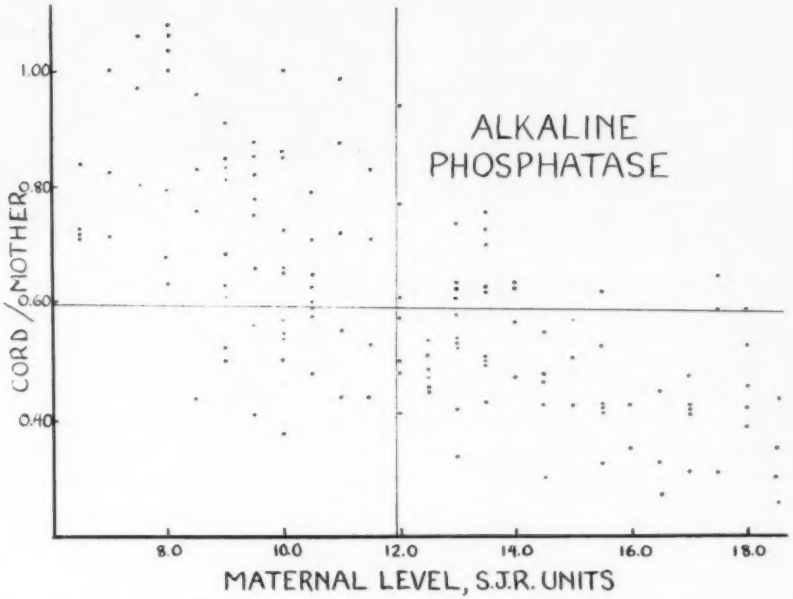

Fig. 3.—Scattergram of mother enzyme level and the corresponding cord/mother ratio for alkaline phosphatase (see Table III).

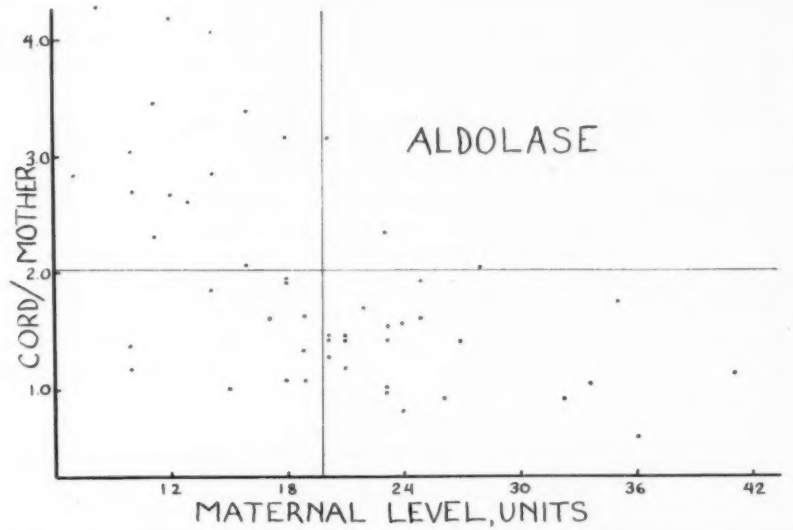

Fig. 4.—Scattergram of mother enzyme level and the corresponding cord/mother ratio for aldolase (see reference 1).

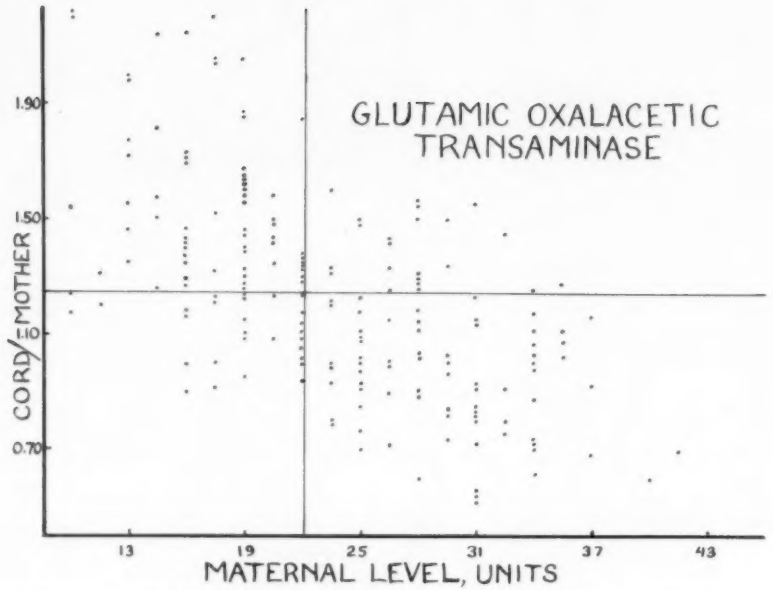

Fig. 5.—Scattergram of mother enzyme level and the corresponding cord/mother ratio for glutamic oxalacetic transaminase (see Table V).

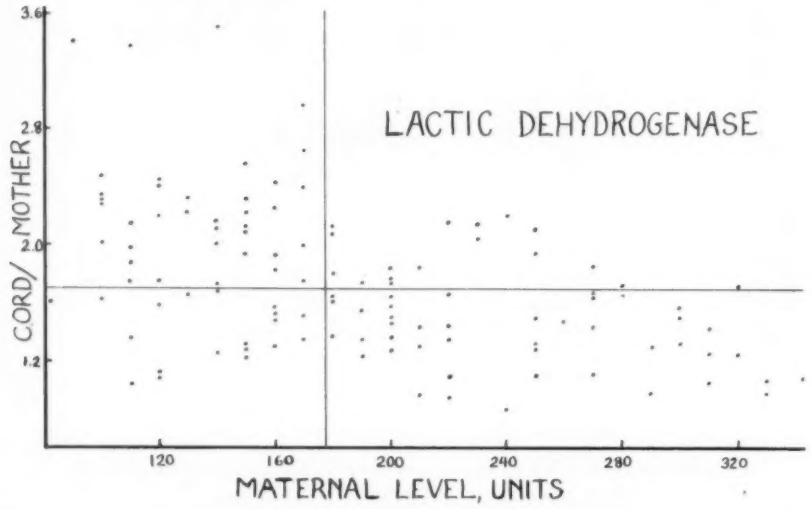

Fig. 6.—Scattergram of mother enzyme level and the corresponding cord/mother ratio for lactic dehydrogenase (see Table VI).

The findings for the 6 enzymes are summarized in Table VII.

TABLE VII. SUMMARY OF ENZYMES IN MOTHER AND CORD SERA

ENZYME	MOTHER	CORD	t
Cholinesterase	0.605 ± 0.092	0.576 ± 0.095	2.88
Tributyrinase	0.607 ± 0.115	0.566 ± 0.115	3.19
Alkaline phosphatase	11.9 ± 3.4	7.0 ± 1.6	16.9
Glutamic oxalacetic transaminase	22.0 ± 6.7	27.3 ± 8.2	5.28
Lactic dehydrogenase	177 ± 67	300 ± 109	8.15
Aldolase	19.6 ± 7.8	40.1 ± 18.1	7.87

Figs. 1 to 6 are scattergrams with the mothers' serum enzyme level as the abscissa and the corresponding cord/mother ratio as the ordinate. The perpendicular intersecting lines in the graph are drawn at the mothers' average enzyme level as a vertical line parallel to the y-axis, and the average cord/mother ratio as a horizontal line parallel to the x-axis. The graphs demonstrate that there is no relationship between the maternal and cord (infant) levels, for if such were the case the points would be concentrated in a horizontal zone along the average cord/mother line. Where the mothers' enzyme level was significantly below or above the average for the whole group, the corresponding cord level did not follow in the same range. Therefore, most of the points lie in the upper left and lower right quadrants.

DISCUSSION

We have been able to classify the 6 enzymes in this study of mother and cord serum into two groups: (1) the esterases which include cholinesterase, tributyrinase, and alkaline phosphatase and (2) those serum enzymes which are elevated in tissue necrosis in adults. The latter group includes aldolase, glutamic oxalacetic transaminase, and lactic dehydrogenase. In the first group, the esterases, the levels are higher in the mother than in the newborn, while the reverse is seen with the other enzymes.

West and Zimmerman[11] found transaminase and lactic dehydrogenase serum levels higher in the newborn when compared to the mother during pregnancy and in labor. Glendening and associates[12] also noted higher whole blood transaminase levels in the cord. Wolfson and Williams-Ashman[13] recorded higher activities of isocitric dehydrogenase in the newborn sera than in the mother at term. In the case of the esterases, Speert and associates[14] showed that the alkaline phosphatase activity of the infant's cord serum was lower than that of the mother's serum.

The placental barrier appears to separate the maternal from the fetal enzyme systems so that different levels exist in mother and fetus. The placenta itself can, however, be a source of the enzymes and many of the known enzymes have been identified in placental tissue.[15]

With the possible exception of alkaline phosphatase, no reasons could be given for the differences of enzyme activities in mother and newborn. It would seem reasonable to assume that the osseous activity in the mother at the end of pregnancy is at a higher level than in the fetus, hence the higher alkaline

phosphatase levels in the mother. With the other esterases, cholinesterase and tributyrinase, the reasons for the higher activity in the mother are less apparent.

Of those enzymes in which the activities are higher in the cord serum, aldolase and lactic dehydrogenase are concerned with carbohydrate metabolism at the 6 and 3 carbon atoms, respectively. Transaminases are concerned with amino acid synthesis, protein metabolism, and only indirectly with carbohydrate metabolism. The higher activity of these enzymes in the fetal serum may be a reflection of the different metabolic states in mother and fetus.

The findings that the enzymes which are elevated in the fetal serum are also those which are higher during necrotic processes in the adult may be of significance, or merely coincidental. We have previously noted[1] that there was no known tissue breakdown occurring at birth that could account for the elevated aldolase in the fetal serum, and the same assumption may be made for transaminase and lactic dehydrogenase in this study. It might be more likely that an active metabolic and growth process is responsible for the higher enzyme levels in the fetus rather than tissue destruction. Elevated serum aldolase[16] and elevated lactic dehydrogenase[17] have been reported in neoplastic disease. West and Zimmerman,[18] in a study of lactic dehydrogenase in malignant disease state that "the elevated levels of this enzyme may reflect the mass of rapidly growing highly glycolytic tissue." On that basis one may also postulate that the enzyme increase in the fetus may be the result of an extensive growth process as compared to adults.

The enzymes that are elevated in the fetal circulation are those that are known to occur in very high concentration in the erythrocytes. The red cells contain aldolase, transaminase, and lactic dehydrogenase in higher orders of magnitude than does the serum. Therefore one must consider as a cause of the higher levels the possibility of excessive red cell destruction and intravascular hemolysis in the fetal circulation. Smith[19] states that "although hemolysis plays a part in the production of neonatal bilirubinemia and icterus, its role is not so outstanding as has often been supposed nor is the hemolysis of neonatal life the dynamic process often described." Mollison[20] found that destruction of erythrocytes transferred from adults into newborn infants proceeded at a standard rate even when the infant was rendered polycythemic by transfusion. Furthermore, Zimmerman and associates[21] found lactic dehydrogenase abnormally elevated in the serum of patients with the hemolytic state of sickle-cell anemia while the serum enzyme was normal in most of the patients with hemolytic anemia. They explained this difference by the reasoning that in sickle-cell anemia erythrocyte destruction is intravascular while in the hemolytic anemias the destruction occurs in the spleen. This would account for the different lactic dehydrogenase activities. No evidence can be found for any unusual intravascular hemolytic processes in the normal fetus and one may conclude also that the elevated enzyme levels in the cord sera at birth are not the result of hemolysis.

The scattergrams (Figs. 1 to 6) indicate that there is no relationship between the individual mother and the cord serum enzyme activity of her infant.

High, average, or low levels in the mother did not reflect corresponding levels in the cord blood of her infant. The scattergram points fall in a hyperbolic fashion rather than a zone parallel to the x-axis. The latter would be the case if the mother and cord enzymes were in the same magnitude. The scattergram figures are similar for the 6 enzymes investigated, even though 3 of the enzymes show cord levels higher than the mother and the reverse in the other 3 enzymes.

SUMMARY

Enzyme activities were determined in cord sera and in the sera of mothers at the time of delivery.

The enzymes studied were grouped into esterases (cholinesterase, tributyrinase, and alkaline phosphatase), and those enzymes that are known to be elevated in necrotic processes (aldolase, glutamic oxalacetic transaminase, and lactic dehydrogenase). In the first group, the esterases, the activities were found to be higher in the mothers' sera, while the reverse was seen with the other enzymes. It is suggested that the elevated levels of aldolase, transaminase, and lactic dehydrogenase in the newborn are not the result of tissue breakdown.

There appears to be no relationship between the individual mother and the cord for any of the enzymes studied.

The possible significance of the results is discussed.

We wish to thank the Department of Obstetrics and Gynecology for cooperating in this study. The technical assistance of Isaac A. Ford and Michael E. Friedman is gratefully acknowledged.

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SERUM TURBIDITY FOLLOWING A FAT MEAL AS A TEST OF MALABSORPTION

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WITH better definition of the causes and treatments of the various malabsorption syndromes, their recognition has become increasingly important.[1, 3] The most commonly recognized defect in absorption involves fat and is manifested as steatorrhea.[2] Unfortunately, present methods of detecting steatorrhea are involved or require special equipment and techniques not readily available.[3] In 1956, Gardner and Santiago[4] described a butterfat tolerance test and evaluated it in normal persons and in patients with steatorrhea due to tropical sprue. Their test was simple, required no involved nor special techniques, and appeared suitable for use as a screening procedure for malabsorption.

This study was designed to evaluate a similar test having somewhat different criteria and using a fat dose related to body weight in a variety of malabsorption syndromes and diseases with known alterations in alimentary lipemia.

MATERIALS AND METHODS

An initial blood serum sample was obtained from the fasting subject. A test meal was then eaten which consisted of 0.5 Gm. per kilogram of body weight of commercial butter, two pieces of toast, 100 ml. of orange juice, and a cup of black coffee without sugar. Subsequently, blood serum samples were obtained at hourly intervals for 5 hours. The optical density of each postcibum serum sample was determined in a Beckman DU spectrophotometer at wave length 620 mμ using the fasting serum as a "blank."

Based on the results in the normal group, an increase in optical density greater than 0.1 in any postcibum sample was chosen as the "normal" value.

RESULTS

One hundred and forty-six tests were done in 121 persons.

The control group included 49 subjects without known gastrointestinal disease (Fig. 1). Eighteen were nonhospitalized volunteers without evidence of any disease. The remaining 31 were patients with diseases not involving the gastrointestinal tract. Diagnoses included polycythemia vera, arteriosclerotic

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cardiovascular disease, rheumatoid arthritis, osteoarthritis, anxiety reaction, bronchiectasis, and pulmonary tuberculosis. There were 20 women and 29 men ranging in age from 15 to 81.

Also tested were 23 hospitalized patients with diseases involving the gastrointestinal tract but without evidence of malabsorption as judged by other parameters of intestinal function. Gastrointestinal x-rays, vitamin A tolerance tests, glucose tolerance tests, tests of vitamin B_{12} absorption, iron tolerance tests, and I^{131}-tagged fat absorption tests were utilized, but not every member of this group had all of these tests (Fig. 1). Diagnoses in this group included psychophysiologic gastrointestinal reactions with diarrhea, ulcerative colitis, postgastrojejunostomy state, peptic ulcer, postcholecystectomy, and acute amebiasis. Ages ranged from 14 to 81, and 9 of the group were women.

The above two groups were combined in the tabulation as the "normal" group (Fig. 2). Eight of the combined (normal) group failed to develop a postprandial lipemia with an optical density change greater than 0.1. No other abnormal parameters of absorption were found in 5 of these 8 subjects who were investigated further (Table I).

TABLE I. RESULTS OF TESTS IN 5 OF 8 PERSONS WITHOUT EVIDENCE OF MALABSORPTION BUT HAVING ABNORMALLY LOW SERUM TURBIDITY FOLLOWING THE FAT TEST MEAL (N = NORMAL)

PATIENT	FECAL I^{131} TRIOLEIN EXCRETION	VITAMIN A TOLERANCE TEST	GLUCOSE TOLERANCE TEST	IRON TOLERANCE TEST	VITAMIN B_{12} ABSORPTION
1. H. P.			N	N	N
2. F. M.		N			N
3. W. H.	1%				
4. K. K.	2%				
5. G. M.	7%				

Twenty-five patients with intestinal malabsorption were tested (Fig. 2). Their ages ranged from 29 to 73 years, and 11 were women. Diagnoses in this group included nontropical sprue, regional enteritis, chronic pancreatitis, complete common bile duct obstruction, extensive small bowel resection, diabetic splanchnic neuropathy, and lymphoma involving the small bowel. Malabsorption was documented as depicted in Table II. None of the patients in this group developed lipemia with an optical density greater than 0.1 after the test meal.

Eighteen patients with diffuse hepatic disease were tested (Fig. 3). Their ages ranged from 16 to 74, and 8 were women. Patients were placed in this group on the basis of liver function tests and biopsy findings, and the diagnoses included portal cirrhosis, biliary cirrhosis, acute hepatitis, and multiple metastases to the liver. Seven of these patients had an abnormal response to the fat test meal. In general, the latter had more severe hepatic disease, although jaundice was not always present.

In addition, 4 of 6 patients with thyrotoxicosis had an abnormally low serum turbidity following the fat meal (Fig. 3). Their ages ranged from 34 to 63 years. The diagnosis of thyrotoxicosis was substantiated in each person by radioactive iodine studies.

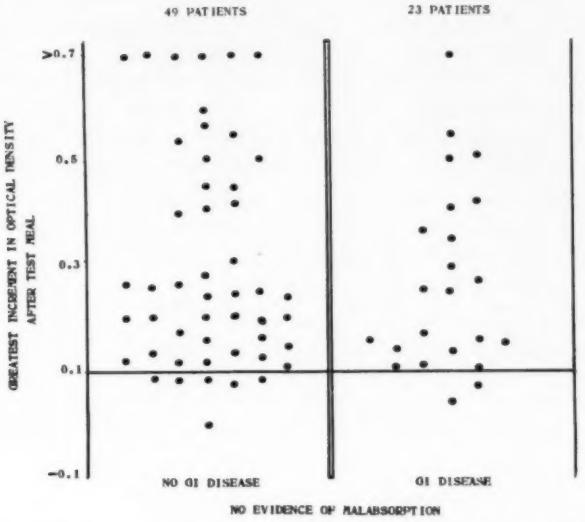

Fig. 1.—Control series. Each dot represents one person. The subjects represented in the left column had no gastrointestinal disease; those on the right had diseases or conditions involving the gastrointestinal tract but did not have malabsorption. The horizontal line at 0.1 is the arbitrary value chosen to separate normal and abnormal.

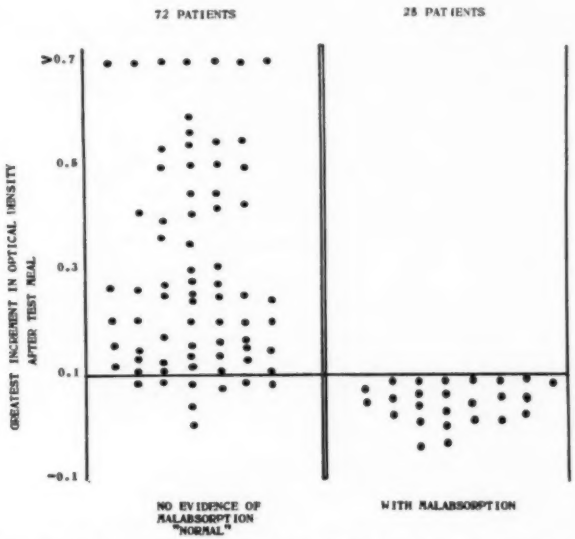

Fig. 2.—Control series versus series of subjects with malabsorption. The two groups in Fig. 1 have been combined in the left column. On the right are depicted results of testing subjects with malabsorption.

TABLE II. EVIDENCES OF IMPAIRED INTESTINAL ABSORPTION IN THE 25 PATIENTS WITH MALABSORPTION, ALL OF WHOM HAD AN ABNORMALLY LOW SERUM TURBIDITY FOLLOWING THE FAT TEST MEAL

PATIENT	CHARACTERISTIC SYMPTOMS	SMALL BOWEL CHANGES BY X-RAY	GLUCOSE TOLERANCE CURVE		ABNORMAL VITAMIN A TOLERANCE	GROSS FAT IN FECES	ABNORMAL VITAMIN B_{12} ABSORPTION	HIGH I^{131} TRIOLEIN EXCRETION	OSTEOMALACIA (CHEMICAL AND X-RAY)	LOW SERUM CAROTENE	PANCREATIC CALCIFICATION	RESPONSE TO GLUTEN-FREE DIET	TISSUE DIAGNOSIS	ULTIMATE DIAGNOSIS
			"FLAT"	ELEVATED										
1. C. H.		X	X		X	X	X	X				X		Idiopathic non-tropical sprue
2. M. C.	X				X			X				X		
3. E. R.	X	X	X		X		X		X	X		X		
4. M. H.	X	X	X		X		X	X	X	X				
5. M. B.	X	X	X		X				X	X		X		
6. T. P.	X	X	X						X	X				
7. E. T.	X		X		X				X	X				
8. J. K.	X			X		X					X			Chronic pancreatitis
9. H. S.	X			X							X			
10. R. W.	X			X		X					X			
11. M. He	X			X									X	
12. A. C.	X			X							X		X	
13. S. R.				X							X			
14. W. K.	X												X	Complete common bile duct obstruction
15. G. Y.	X												X	
16. O. B.	X												X	
17. A. W.	X												X	
18. D. S.	X			X	X		X	X	X	X				Secondary steatorrhea
19. E. D.		X		X	X									
20. B. S.	X				X		X							
21. A. L.		X		X										
22. W. H.						X								
23. A. P.	X		X			X	X						X	
24. C. B.	X		X			X	X		X	X				
25. I. B.	X							X					X	

Five of the healthy persons with a normal rise in serum turbidity were retested and the results were again normal. Ten persons with initially abnormal results were retested. Of these 10 persons, 8 again had abnormal tests. One of these 8 subjects had an abnormal test on 3 occasions. This person (Subject K. K. in Table I) had no signs or symptoms of gastrointestinal, liver, or thyroid diseases. Two persons (whose initial tests were abnormal) had normal responses to the second fat test meal. One of these latter had a hemoglobin of 4.0 Gm. per 100 ml. and a hematocrit of 15 due to iron deficiency at the time of the initial test, but the test was subsequently normal after the hemoglobin had been restored to 16 Gm. and the hematocrit to 48 by iron therapy. Other tests for malabsorption in this patient were normal at the time of the second butterfat tolerance test (Subject H. P. in Table I). It is possible that the accelerated circulation and relatively greater plasma volume accompanying the chronic severe anemia were responsible. The second person was without signs or symptoms of gastrointestinal, thyroid, or hepatic diseases, was not anemic, and other tests of intestinal absorption were normal (Subject F. M. in Table I).

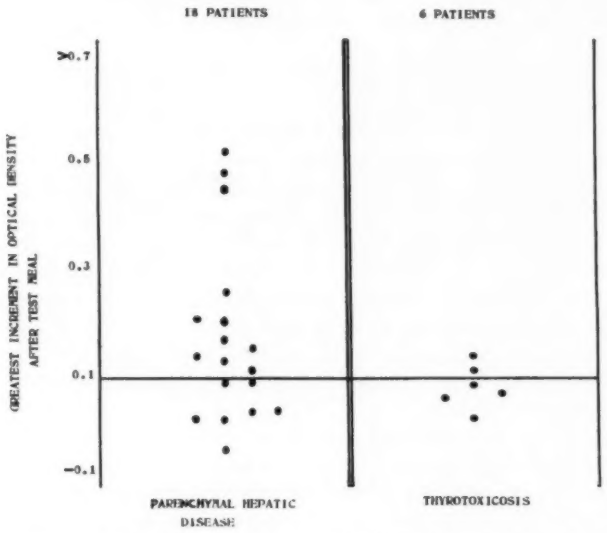

Fig. 3.—Results of fat tolerance tests in patients with parenchymal hepatic disease (left) and thyrotoxicosis (right).

DISCUSSION

Since neutral fat in serum is present principally as minute refractile globules, these may be enumerated in a dark-field chamber and the result recorded as "chylomicron counts."[5-7] The chylomicron counting method has been shown to correlate well with the chemical determination of serum neutral fat in patients with steatorrhea secondary to pancreatitis.[8] Chylomicron counts

correlated well with optical density in a limited number of patients in the same study. This technique, however, is tedious and requires an experienced technician.

Chylomicrons in the aggregate are responsible for alimentary lipemia.[9] It is the latter which is the major determinant of serum turbidity, and thus optical density.[10] Technically, optical density is simple to determine. No critical measurements, reagents, or color developments are required. Although a Beckman DU spectrophotometer was used in this study, theoretically any device capable of measuring optical density would be suitable. The wave length of 620 mμ was chosen to minimize the effect of hemoglobin in the serum.

Butter was used as the test fat because of its acceptability and availability. The dose of 0.5 Gm. per kilogram was arbitrarily chosen as a quantity small enough to be palatable but large enough to challenge the intestine and to produce lipemia. All of the subjects were able to eat the entire test meal, and no adverse effects were observed.

In any oral tolerance test, the amount of the test substance in the blood at any given moment is related to many factors. These include the amount ingested, the physical state of the substance, gastric emptying time, rate and site of intestinal absorption, the blood volume, and the rate of clearing from the blood.

It has been previously demonstrated that simultaneous ingestion or injection of carbohydrate reduces alimentary lipemia.[11] For this reason, the quantity of carbohydrate in the test meal was kept constant. Simultaneous ingestion of protein may also reduce alimentary lipemia.[12]

Clearing of neutral fat from the serum of thyrotoxic subjects and some patients with diffuse parenchymal hepatic disease may be accelerated.[13] Accordingly, reduced alimentary lipemia in patients with liver disease or hyperthyroidism is not necessarily evidence for malabsorption. In general, patients with liver disease who failed to develop normal serum turbidity following the test meal had the more severe disease as judged by the liver biopsy and functional tests. One of the 4 of 6 patients with thyrotoxicosis whose initial test was abnormal was retested when she had become euthyroid following treatment with I^{131}. The test was then normal.

Serum turbidity is less influenced by other variables. Exercise has an inconstant but limited effect on alimentary lipemia.[11, 14] All tested subjects were sedentary but not necessarily at rest nor in bed. One Subject (K. K. in Table I) had an abnormal test when actively working, but retesting during bed rest did not significantly alter the result. Diarrhea not associated with malabsorption, moderately severe anemia, ascites, congestive heart failure, disease of the colon, and hypothyroidism per se were not found to affect the test results.

In the "normal" group (Fig. 2), the fasting serum carotene level and the vitamin A tolerance curve was determined in 6 subjects. Glucose absorption was tested in 11. All of the values were normal; thus, there were no "false positives" attributable to these tests in this study.

Nine vitamin A tolerance tests were done in the 15 patients with malabsorption not due to chronic pancreatitis or common duct obstruction. The values

obtained were all abnormally low and thus there were no "false negatives" attributable to this test. Of this same group, 3 of the 9 tested had normal fasting serum carotene levels. Fourteen of these 15 subjects had glucose tolerance tests. Five of the curves obtained were normal or elevated and thus were "false negatives."

It is not known why a few persons without history or findings of gastrointestinal disease failed to develop the expected serum turbidity following the test meal. In 3 of these subjects, fat absorption was normal as measured by an I^{131}-tagged neutral fat excretion method[15] (Table I). None of these 3 had thyrotoxicosis or hepatic disease.

SUMMARY

One hundred and forty-six determinations of the maximum increase in optical density of the blood serum following a meal containing a known amount of butter were made in 121 persons.

All of the 25 patients known to have malabsorption had abnormal test results. In the absence of thyrotoxicosis or hepatic disease, the measurement of serum turbidity following a fat meal is a simple and useful screening test for malabsorption.

We appreciate the assistance of the Dietary Department of the University Hospitals, Madison, Wis.

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DELAY OF ABSORPTION OF RADIOLABELED CYANOCOBALAMIN IN THE INTESTINAL WALL IN THE PRESENCE OF INTRINSIC FACTOR

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RECENTLY methods have been developed for the measurement of plasma absorption of vitamin B_{12} following oral administration of radiolabeled cyanocobalamin.[1, 2] A relatively rapid influx of radioactivity into the blood was observed in control subjects as well as in patients with Addison's pernicious anemia following large oral test doses,[3] while entirely different results were obtained after small test doses.[1-4] Control subjects showed no or negligible absorption during the first 3 to 4 hours of the tests, followed by a rapid rise in radioactivity and peak concentrations in the 8- to 12-hour interval following doses ranging from 0.46 to 2.0 μg.[1-4] The same was true for patients with Addison's pernicious anemia when intrinsic factor (IF) was administered together with the labeled cyanocobalamin.[1-3] This delay in the rise of the plasma radioactivity after small oral test doses is a rather unusual phenomenon and has received previous comment.[1-3]

The results of radioactivity measurements over the abdomen and liver,[1, 5] as well as studies of the urinary excretion of radioactivity,[6] and of the plasma absorption patterns of peripheral venous blood[1, 2] have all been interpreted as being consistent with a slow absorption of vitamin B_{12} through the intestinal wall. However, alternative explanations such as rapid absorption from the intestine with temporary storage in the liver or other extragastrointestinal organs[1, 2, 5, 6] or an effect of gastrointestinal transit time[1, 2, 5] were not entirely ruled out.

The purpose of the present report is to describe results of studies made to determine more precisely the nature of the delayed plasma absorption of cyanocobalamin following small oral doses in the presence of intrinsic factor.

MATERIALS AND METHODS

Included in this report are the results of absorption studies in 10 control subjects, in 5 patients with portacaval shunts, one of which has been reported previously[3]; in 1

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patient whose portal vein was catheterized; and in 2 patients with ileostomies. The plasma absorption was determined according to a previously described technique.[2, 3] The test doses consisted of 0.56 μg amounts of cyanocobalamin labeled with 0.5 μc Co^{60}* except in one instance where 0.39 μc Co^{60} was used. The doses were administered to the subjects in the fasting state and food was withheld for 2 more hours. Radioactivity of blood samples was measured in 20 milliliter amounts of plasma in a well-type scintillation counter. An automatic vial changer facilitated the use of a pre-set count of 32,000 for each determination. Since 5 counts per minute (c.p.m.) were equal to 3 times the standard deviation of the background count, any figure above 5 was considered significant. Fecal radioactivity was determined in 20 ml. aliquots of homogenized stool. In vivo counting over the liver and abdomen was carried out by the method of Glass and associates[7] as described elsewhere.[3] The abdominal radioactivity figures are the averages of the counts obtained over the lower and upper left abdominal quadrants.

Intrinsic factor was assumed to be present because of the normal types of peripheral blood absorption curves obtained in each instance.

RESULTS

The observed delay in the rise of radioactivity in peripheral blood could result from slow absorption from the intestine or from an effect of the liver, lungs, or peripheral tissues. In order to circumvent the possible role of the liver, the plasma radioactivity patterns were determined in 5 patients with portacaval shunts. All 5 patients had Laennec's cirrhosis of the liver. The portacaval shunting operations had been performed 2 to 56 months before the absorption tests. In all 5 patients the radioactivity curves of the plasma were similar to those of 10 control subjects (Fig. 1). If the livers were effectively bypassed by the portacaval shunts, these results indicated that the liver did not cause the delay in the rise in plasma radioactivity. One of the 5 patients (K. H.) has died since the test was performed and autopsy revealed a widely patent portacaval shunt. The clinical courses of the other 4 patients indicated well-functioning shunts.

In order to exclude the liver more definitely, and at the same time exclude possible effects of the lungs and peripheral tissues, serial blood samples were obtained by catheter directly from the portal vein in 1 subject. The patient was a 29-year-old man who had cirrhosis of the liver of unknown etiology. Because of hemorrhage, apparently from esophageal varices, a portacaval shunt operation was contemplated. However, at laparotomy the portal venous pressure was found only mildly elevated (25 cm. of water) and the shunt procedure was not carried out. A catheter was left in the portal vein for several days to record variations in pressure. On the fifth postoperative day when the patient's gastrointestinal functions had returned to normal, an absorption test was performed. Six samples of blood were obtained serially from the portal vein by means of the indwelling catheter and 2 samples were obtained from a peripheral vein. X-ray examinations before and upon completion of the absorption studies showed the catheter correctly placed. There was a delay in the rise of the plasma radioactivity in the portal vein with highest values at 6 and 8 hours (Fig. 2). The two peripheral blood samples

*Kindly supplied by Dr. N. S. Ritter, Merck & Company, Inc., Rahway, N. J.

showed low activity at 3 hours and higher activity at 8 hours—a normal pattern of absorption. In order to double check on the possibility of an early influx of radioactivity into the portal vein, the absorption test was repeated the next day. Portal vein blood samples were obtained immediately before the repeat oral test dose for a background count and 1 and 2 hours after the dose. These samples confirmed that there was slow absorption into the portal system.

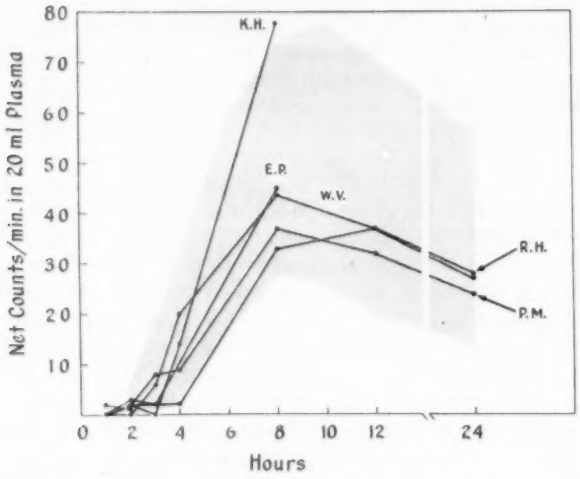

Fig. 1.—Plasma absorption of cyanocobalamin in peripheral venous blood in 10 control subjects (shaded area) and in 5 patients having functioning portacaval shunts (solid lines). Oral test dose 0.56 μg vitamin B_{12} (0.5 μc Co^{60}).

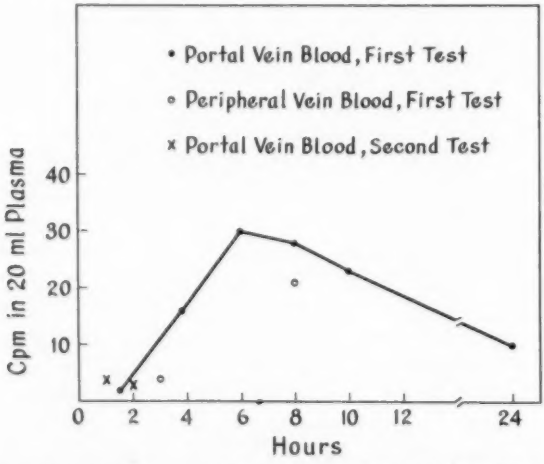

Fig. 2.—Plasma absorption of cyanocobalamin in portal vein blood (solid dots and crosses) and in peripheral venous blood (circles). Oral test dose 0.56 μg vitamin B_{12} (0.39 μc Co^{60}).

The delayed rise in plasma radioactivity found in portal vein blood of this patient and in the peripheral blood of patients with portacaval shunts indicated that slow absorption from the intestine was responsible for the delayed rise in plasma radioactivity routinely found after small oral test doses of radiolabeled cyanocobalamin in the presence of intrinsic factor.

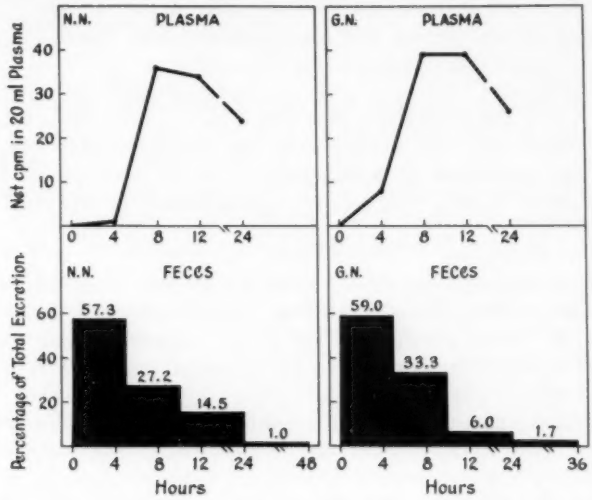

Fig. 3.—Measurements of fecal excretion of radioactivity in 2 patients with total colectomy and ileostomy with simultaneous plasma absorption curves from peripheral venous blood.

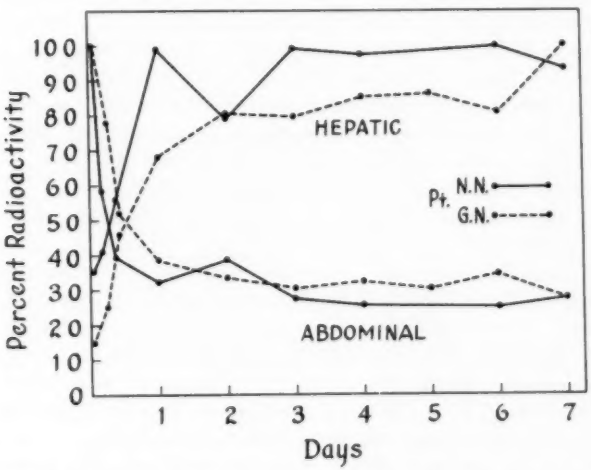

Fig. 4.—In vivo radioactivity measurements over the hepatic and abdominal areas in 2 patients with total colectomy and ileostomy. The abdominal radioactivity is the average count obtained over the upper and lower left quadrants. Oral test dose 0.56 μg vitamin B_{12} (0.5 μc Co^{60}).

Slow absorption from the intestine could result either from a delay in transit of the test dose to an area or areas of the intestine where absorption occurs, or from slow transport through the intestinal wall itself. In order to determine which mechanism prevails, absorption studies were made in 2 patients who had ileostomies because of total colectomy for ulcerative colitis (Fig. 3). In each case the peripheral blood showed the usual delay of rise in the plasma radioactivity. However, in both instances the bulk of the radioactivity which was excreted had already passed through the entire small bowel and into the ileostomy bag within the first 5 hours.

In vivo hepatic and abdominal radioactivity measurements were made in these 2 patients and the results are shown in Fig. 4. After the rather rapid evacuation of radioactivity into the ileostomy bags during the first day, in each patient there was persistence of radioactivity over the abdomen with only a gradual rate of fall. During this time hepatic radioactivity increased.

DISCUSSION

These studies provide more direct evidence than heretofore available concerning the delay of absorption of small doses of radiolabeled cyanocobalamin in the presence of intrinsic factor. They show that under these conditions cyanocobalamin is stored temporarily in the intestinal wall with slow release to the blood stream.

The radioactivity measurements of serial blood samples obtained directly from the portal vein were of particular note. There was no measurable radioactivity during the first 2 hours of the tests followed by a rise in plasma radioactivity, similar to the curves obtained from peripheral blood. This demonstrated that slow absorption from the intestine caused the delay of the rise of radioactivity found in the peripheral blood. The observations in the patients with portacaval shunts were in agreement with this conclusion.

The peak of radioactivity of the portal vein blood was at 6 hours, while it is usually found in the 8- to 12-hour interval in the peripheral blood. However, there were only 2 c.p.m. difference between the 6- and 8-hour samples of portal blood. This difference is not statistically significant. Moreover, 1 of the 10 control subjects had equal radioactivity values in the 6- and 8-hour peripheral samples. The most important characteristic, i.e., the absence of radioactivity during the first hours of the tests, was similar for both portal and peripheral blood curves.

Of interest was the fact that the portal vein blood radioactivity was significantly higher than that found in a peripheral blood sample obtained at the same time. This discrepancy has been found in dogs[8] and by us in other patients.[9] The difference may result from deposition or utilization of some of the radioactive vitamin B_{12} in the liver, lungs, or peripheral tissues or may result simply from a dilution effect since the portal venous blood volume is much smaller than the total blood volume of the body.

The studies in the 2 patients with ileostomies were necessary to define the nature of the delayed absorption from the intestine. It was found that the major portion of the radioactivity excreted from the small intestine appeared

in the ileostomy bags before the peak of radioactivity in the peripheral blood. Although these observations do not reveal the specific site or sites of absorption, they eliminate transit time as the factor responsible for delayed absorption. At the same time, the observation of persistence of abdominal radioactivity with a slow increase in hepatic radioactivity is consistent with temporary storage of cyanocobalamin in the intestinal wall resulting in delayed and prolonged release to the blood.

SUMMARY AND CONCLUSIONS

1. This study was made to determine the approximate locus of delay of absorption into the plasma of small doses of radiolabeled cyanocobalamin in the presence of intrinsic factor.

2. Absorption curves obtained from peripheral blood in 5 patients with portacaval shunts and from the portal vein blood of 1 subject showed delays of the peak plasma radioactivity consistent with slow absorption from the intestine.

3. Observations were made in 2 patients with ileostomies and colectomies to ascertain the cause of the retarded intestinal absorption. Maximum excretion of radioactivity into the ileostomy bags occurred before peak radioactivity appeared in the peripheral blood. On the other hand, radioactivity measurements in vivo showed persistence of abdominal radioactivity with a gradual rise in hepatic radioactivity.

4. It is concluded that the delayed plasma radioactivity peak under the conditions of these studies results from slow intestinal absorption which is due to temporary storage of cyanocobalamin in the intestinal wall.

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GLOMERULAR PERFUSION DURING ACUTE RENAL INSUFFICIENCY FROM MERCURY POISONING IN THE RAT

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OVER the past two decades much progress has been made in understanding the pattern of structural damage and of functional derangement in the syndrome of reversible acute renal failure which may result from such diverse causes as hemorrhage, burns, crushing injury, shock, and a variety of nephrotoxins. Much evidence has accumulated which indicates that at the time of the initial insult to the kidney, renal blood flow is sharply diminished. The definitive studies of Oliver and associates[1] employing microdissection of entire nephrons have shown that in a variety of renal lesions in which an endogenous or exogenous substance toxic to the kidney is involved there is tubular necrosis often sharply localized to tubular cells in a particular portion of the nephron. In addition, in all fatal human cases of such poisoning and in severe poisoning in experimental animals there is disruptive tubular damage or tubulorhexis, spotty in distribution and thought to be due to ischemia from reduced renal blood flow. There has been, however, considerable divergence of opinion as to how long the reduction in renal blood flow persists and more particularly as to whether such a reduction is a major and necessary factor in producing the associated oliguria or anuria.

In the present study, potentially reversible acute renal insufficiency was produced in the rat by the administration of mercuric chloride by a technique similar to that of Oliver,[2] which is designed to produce chiefly nephrotoxic damage. The perfusion of the renal glomeruli was studied by the technique of Schlegel, in which small blood vessels are visualized by means of a fluorescent dye. The glomerular perfusion at various intervals after the chemical insult has been correlated with the degree of oliguria produced and with the histologic damage.

MATERIALS AND METHODS

Albino rats of the Sprague-Dawley strain weighing between 170 and 310 Gm. with an average of 223 Gm. for the controls and 269 Gm. for the experimental animals were used throughout the experiments.

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The distribution of renal blood flow was studied by the method of Moses and Schlegel[3] by the injection of thioflavin-S, a fluorescent dye which attaches itself to the intima of the blood vessels through which it passes and which may be visualized under ultraviolet light. In the concentrations used the dye has no direct physiologic effect on the small blood vessels.[4]

Rats used for the studies were kept in metabolism cages and, to obtain a high rate of urine flow, were given only 7 per cent dextrose in water as a source of nourishment for 48 hours before the experimental procedure and thereafter. This ensured a urine flow of from 34 to 110 ml. per day with an average of 80 ml. The glomerular perfusion of the control animals was then visualized by the following method. The animals were anesthetized with 0.4 mg. per kilogram bodyweight of sodium pentobarbital given intraperitoneally. A midline incision was made in the abdomen and one of the femoral veins was exposed in the inguinal region. The wounds were covered with pads moist with saline, and 20 minutes was allowed for a return to equilibrium, after which 0.3 ml. of 4 per cent thioflavin-S in saline was injected intravenously. Thirty seconds after the injection the hilum of the right kidney was clamped and the organ dissected free. It was later found that comparable and technically more satisfactory results in normal and experimental rats were obtained if the hilum of the kidney was pinched without compressing the parenchyma and the organ rapidly avulsed. The kidney was then weighed, sectioned in the median longitudinal plane, and immersed in glycerin. The cut surface was then pressed firmly to a glass slide to maintain it in a single plane and examined by incident ultraviolet light from a mercury arc lamp with 80 per cent of its emission between 3,600 and 3,700 Å. In an initial series of rats, photographs were made of the entire section of cortex. However, it was found difficult to obtain photographs in which all of the fluorescent glomeruli consistently remained in the plane of focus. For this reason the technique of Hartman and Flagg[5] was used, with slight modifications, in which direct counts were made of the glomeruli by means of a micrometer eyepiece fitted with a ruled grid and a yellow filter to eliminate potentially harmful ultraviolet light. In each kidney a total of 25 squares, each slightly under 1 mm.^{2} in area, was counted along the subcapsular region. Repeat counts of glomeruli in normal kidneys were found to be reproducible within 5 per cent and in damaged kidneys within about 10 per cent. Histologic sections were made of all kidneys, using both hematoxylin-eosin and Mallory's trichrome stains.

The capsular and medullary tissue of normal kidneys exhibit a strong natural fluorescence under ultraviolet light and areas of necrotic tissue in damaged kidneys likewise fluoresce. However, the glomerular tufts were not visible in such severely damaged kidneys when no thioflavin was injected. The kidneys subjected to mercury poisoning were edematous, as indicated by an increase in weight per 100 Gm. of rat weight of from 0.86 to 1.10 Gm. This edema increased the area of the cut surface of the kidney, thus reducing the glomerular counts per unit area. However, the length of the normal kidney is reduced 9 per cent as a result of dehydration by immersion in glycerin. It is probable that the edematous kidney is even more greatly contracted by this dehydration, although measurements were not made to determine this point. Since a number of uncertainties are involved in any attempt to correct for these factors, and since the results would not be materially affected, no such correction has been attempted. All of the counts were made by one person (B. S.), while frequent counts were checked by a second observer.

Acute renal insufficiency was induced in the experimental animals by subcutaneous injection in the flank of 10 mg./Kg. of bichloride of mercury, a dose previously found[2] to induce anuria or severe oliguria in a high percentage of rats while fatally poisoning only a small per cent.

In a number of animals with chronic renal lesions, blood urea nitrogen determinations were made by the method of Gentzkov.

RESULTS

In Fig. 1 is shown a median longitudinal section of the renal cortex from a normal rat in which the glomeruli are sharply visualized by means of fluorescent dye. In our series of 18 normal control male rats averaging 223 Gm., an

average of 18.5 ± S.D. 1.1 glomeruli per mm.^{2} was visualized. These counts are considerably lower than that of 25.3 glomeruli per mm.^{2} reported by Hartman and Flagg, using an essentially similar technique.[5] This is explained by the fact that their rats were smaller, ranging from 60 to 160 Gm.

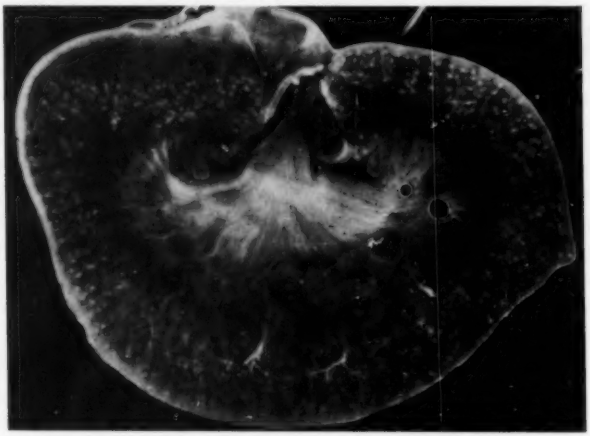

Fig. 1.—Median longitudinal section of the kidney of a normal rat viewed by ultraviolet light following injection of thioflavin-S 30 seconds prior to removal. The capsule and the medullary structures are naturally fluorescent. (Magnification ×20; reduced ⅔.)

Fig. 2.—Median longitudinal section of the kidney of a rat 48 hours after administration of 10 mg./Kg. of mercuric chloride. Output of urine was 1 ml. during the 24 hours prior to study. Technique as in Fig. 1. (Magnification ×20; reduced ⅔.)

It was found that a subcutaneous dose of 10 mg./Kg. of bichloride of mercury in these young mature rats was sufficient to produce oliguria or anuria in a majority of the rats; 10 per cent were killed, and a diuretic response was produced in 12 per cent. This response is consistent with that found by Oliver,[2] employing the same dose of mercuric sublimate.

Fig. 2 is a photomicrograph of the cortex of the kidney of a rat with severe oliguria resulting from administration of mercuric chloride 2 days previously. This animal had developed marked oliguria with a urine volume of only 4 ml. the first day and 1 ml. the second day after the injection. When compared with the normal cortex of Fig. 1, there is evident little if any reduction in the number of perfused glomeruli.

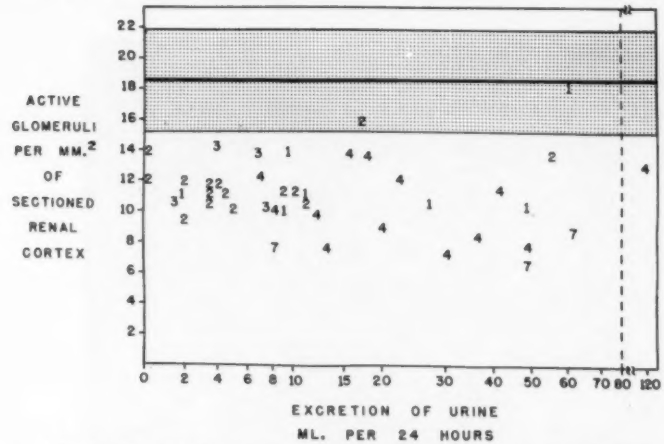

Fig. 3.—The relation of urine flow to the number of active glomeruli demonstrable by use of thioflavin-S in rats given mercuric chloride 10 mg./Kg. The output of urine during the day preceding the glomerular count is graphed on a log scale. The vertical dashed line represents the average urine output prior to giving mercuric chloride. The heavy horizontal line represents the mean glomerular count of 18 normal rats and the stippled area 2 standard deviations above and below this mean. The numerals marking the values for each rat indicate the number of days which had elapsed subsequent to giving the mercuric chloride.

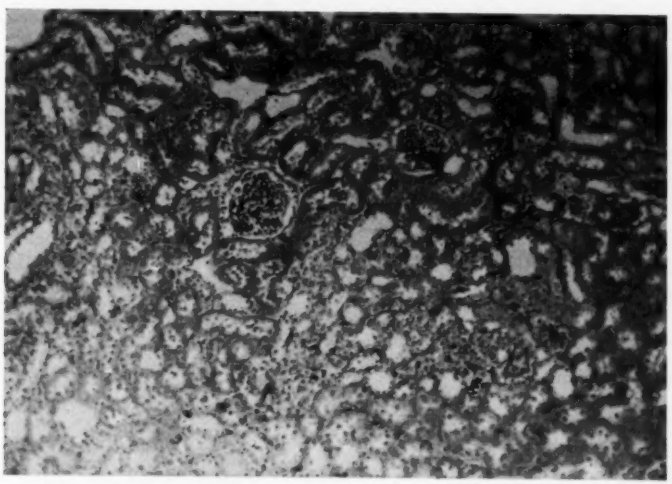

Fig. 4.—Section of the cortex of the kidney from a rat 72 hours after administration of mercuric chloride 10 mg./Kg. This rat had a diuretic response of 55, 60, and 120 ml. of urine on successive days. There were 13 perfused glomeruli per square millimeter of sectioned cortex. (Magnification ×80; reduced ⅝.)

In a series of 48 rats given mercuric chloride, counts of the number of perfused glomeruli in 44 surviving animals were compared with those of normal rats. The experimental animals considered as a group had a majority of the glomeruli actively perfused, a mean of 11.1 ± 2.4 as opposed to 18.5 ± 1.05 in the experimental group. The counts in the experimental kidneys are not corrected for edema secondary to administration of the mercuric chloride. If such a correction were made, it would further minimize the difference.

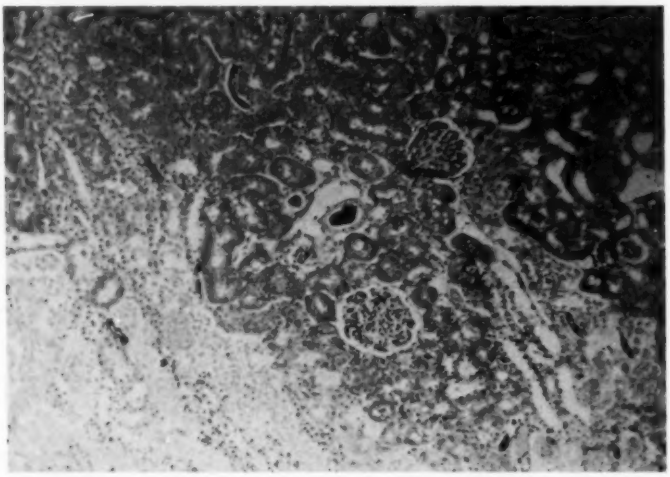

Fig. 5.—Section of the cortex of the kidney from a rat 96 hours after administration of mercuric chloride 10 mg./Kg. Urine flow was 0 and 4 ml. on successive days. The technique was the same as in Fig. 4. There were 14 perfused glomeruli per square millimeter of sectioned cortex.

The diagram in Fig. 3 shows the relation between the number of visualized active glomeruli both to the urine flow and to the length of time following administration of mercuric chloride. No correlation was found between the period of time following the injection of the mercury, whether 1, 2, 3, 4, or 7 days and the number of glomeruli actively perfused. Likewise, no correlation was found between the number of glomeruli actively perfused and the volume of urine excreted during the 24 hours before the counts were made.

On histologic examination varying degrees of damage ranging from cloudy swelling to tubular necrosis were noted. A low-power view of a kidney examined on the fourth day after administration of mercury and showing minimal tubular damage is shown in Fig. 4. The glomeruli are intact and filled with red blood cells, which stain brilliantly with Mallory's trichrome stain. Moderately well-preserved loops of proximal convolutions are visible. Other tubular cells show a loss of affinity for the stain, but no gross disruption or necrosis. The animal from which this kidney was taken did not develop oliguria following the injection of mercury and on the fourth day had a diuresis of 120 ml. per day. In this particular animal, the glomerular count was 13 per mm.^{2} of the sectioned cortex as compared with the normal mean of 18.5. In Fig. 5 is

shown a low-power view of the kidney from a rat examined on the fourth day following administration of mercury. This rat was severely oliguric with volumes of urine of 7, 0, and 4 ml. per 24 hours on the 3 successive days. The cells of the proximal convolutions are swollen, but still darkly staining and may be viable, but other cells have both their cytoplasm and nuclei so disrupted as to be unrecognizable. In addition, there are eosinophilic casts within a number of the tubules. The glomerular capillaries, however, appear normally filled

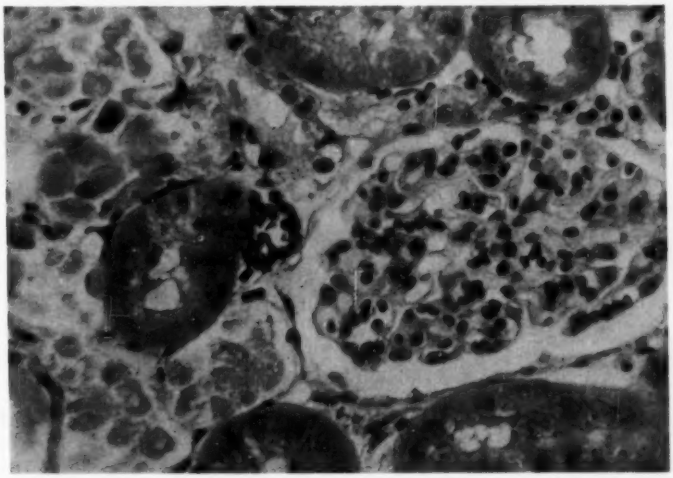

Fig. 6.—High-power view of the glomerulus from the kidney of a rat with 24-hour urine volumes of 4 and 0 ml. 48 hours after administration of $HgCl_2$ 10 mg./Kg. Mallory's trichrome stain. (Magnification ×400; reduced ⅝.)

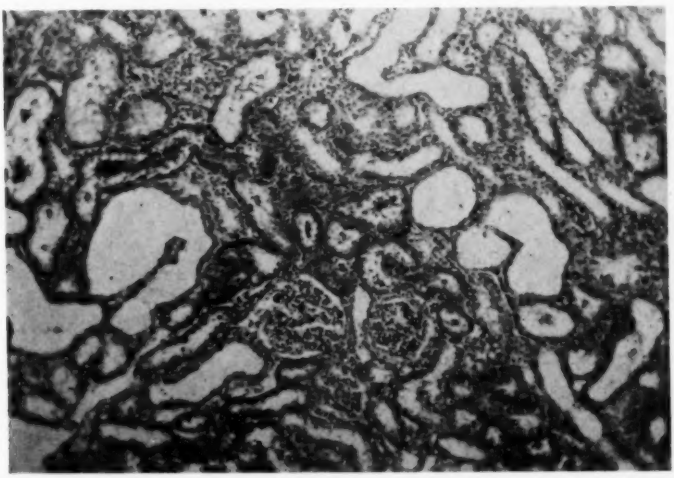

Fig. 7.—Low-power photomicrograph of the renal cortex of a rat 7 days after the administration of mercuric chloride 10 mg./Kg. There were 9 perfused glomeruli per square millimeter of sectioned cortex. (Magnification ×80; reduced ⅝.)

with red blood cells. The glomerular count in this severely damaged kidney was 14 per mm.^{2} which is close to the normal range of 18.5 ± S.D. 1.1. In Fig. 6 is shown a high-power view of damaged tubular cells in juxtaposition with a normal-appearing glomerulus from a severely anuric rat. This lack of correlation between the degree of histologic damage to the renal tubular cells and both the histologic appearance of the glomeruli and their perfusion with the fluorescent dye was seen throughout the entire group of sections.

Three rats were examined 7 days after administration of mercuric chloride. One remained oliguric and two entered a diuretic phase, but had slight azotemia at the time of examination. Histologic sections showed apparent enlargement of the lumen of many nephrons with regeneration of darkly staining, flat, atypical tubular epithelium with considerable distortion of the normal architecture. In Fig. 7 is shown a typical field in a rat with a glomerular count of 9 per mm.^{2} and a blood urea nitrogen of 23.

DISCUSSION

Over a period of years a number of theories have been advanced to explain the anuria of acute renal insufficiency. Over 30 years ago Dunn[6] assumed that the oliguria could be explained by back-diffusion through the grossly damaged tubular epithelium, and since then others have advanced the same explanation (Richards,[7] Corcoran and associates,[8] Bywaters and Dible,[9] Redish and coworkers,[10] Marshall and Hoffman,[11] and Friedman and associates.[12]

There are conflicting reports of renal blood flow in acute renal insufficiency resulting from nephrotoxic agents. Because of the low extraction ratio of paraaminohippurate (PAH) in the presence of tubular necrosis, estimation with PAH is difficult even if renal vein catheterization is performed and the Fick principle applied. Sirota[13] has reported two relatively normal and two markedly reduced values for renal plasma flow in patients poisoned with carbon tetrichloride when studied from 8 to 27 days after onset of oliguria. Bull[14] reported measurements of renal plasma flow by use of PAH and renal vein catheterization in 6 patients with acute tubular necrosis from the third to the seventeenth days and found all to be below 150 ml. per minute. On the basis of these measurements and others by conventional clearance techniques he postulated that during the phase of anuria renal blood flow is minimal and that not until return of renal blood flow to near normal levels is the early diuretic phase initiated. In more recent studies, renal blood flow has been measured by employing the Fick principle and the diffusion into the kidney of an inert gas. The finding of Conn and associates[15] that marked renal circulatory derangements in dogs were not necessarily concomitant with tubular damage produced by mercury is consistent with that of the present study in rats. Employing nitrous oxide, they found that the lower blood flow obtained in 4 surviving dogs was 155 ml. per 100 Gm. of renal tissue, and the highest was 400 ml., as compared to their normal range of 290-400 in control animals. Prerenal factors may have contributed to the reduction of renal blood flow, since all the experimental animals were suffering from dehydration and bloody diarrhea. Renal

edema was not noted. Somewhat similar results were obtained by Brun and co-workers[16] who measured renal blood flow with radioactive krypton[85] in patients with acute renal insufficiency from barbiturate poisoning. In general, renal blood flow and oxygen consumption were reduced to 20 and 25 per cent, respectively. Those subjects with a severe reduction of renal blood flow were at the same time anuric. Nevertheless, one subject with a reduction in renal blood flow of only 30 per cent was as severely anuric as those with reduced flows.

In considering possible mechanisms of anuria, Oliver and co-workers[1] point out that a constellation of interrelated factors must be involved in any given instance. It seems probable that the pattern of these factors varies with different etiologic types of acute renal insufficiency and at different stages of their progression. These authors list 6 factors: renal ischemia during the early stages of the syndrome, increased permeability of the tubular wall and back-diffusion, outpouring of tubular fluid in toto through holes in disrupted tubules at every level of the nephron, vascular and tubular stasis from rising intrarenal pressure, peritubular inflammatory reaction, and mechanical stoppage of tubules with casts. In a later publication, Oliver[2] concludes from morphologic evidence and also from the clinical functional studies then available that during the oliguric phase of acute tubular necrosis, there is persistence of the reduced renal blood flow after the initial insult, plus loss of proximal tubular function, and that the anatomic counterpart is glomeruli with intact but empty capillary tufts, tubulorhexic and nephrotoxic tubular lesions, and increased intrarenal tension. The present studies of renal damage due to mercury poisoning have dealt with an experimental lesion in which the toxic agent is given subcutaneously so that it only gradually reaches the kidneys. As shown by Oliver,[2] this produces predominantly nephrotoxic damage to the proximal convolutions and the lesions are not comparable to those lesions in which the factor of patchy ischemia is more marked and in which tubulorhexic lesions predominate. However, the insult was severe enough to produce death in about 10 per cent of the animals and severe oliguria in the majority. The findings in this experimental model point to a different balance of factors during the phase of anuria or oliguria. Since glomerular perfusion is largely preserved and bears no direct relation to the urine flow, a reduction in glomerular perfusion cannot be considered the major cause of the oliguria. One cannot directly equate perfusion of glomeruli, as demonstrated by staining of the intima by the fluorescent dye, with renal blood flow. However, since the kidneys were removed after only a few circulation times, the counts of perfused glomeruli provide an index of flow sufficient to exclude severe reduction of blood flow to particular segments of the kidney. It seems likely that the functional defect from this particular lesion in the rat may be similar to that noted by Richards[7] in the frog. By direct observation and micropuncture of individual nephrons of frogs poisoned with mercuric chloride, he demonstrated, in 1929, that "... fluid is being separated from the blood within the Malpighian bodies, not at a slower rate than normal, but at a faster rate. ... Its composition is normal, but no drop of urine issues from the ureter." We are inclined to agree with his conclusion, reached

30 years ago, that ". . . under these abnormal conditions the osmotic pressure of the blood proteins is unobstructed by the normal quantities of tubular epithelium and is able to draw all or nearly all of the glomerular filtrate back into the blood stream." However, it should be emphasized that this conclusion cannot be extended by analogy to other experimental circumstances or to clinical cases of acute tubular necrosis in man.

One characteristic of the healing phase of acute tubular necrosis is the rapidity with which damaged tubular epithelium regenerates.[2] Since essentially all the blood supplying the tubules first traverses the glomeruli, it seems logical to assume that such healing could take place readily during the period of anuria and oliguria only if the glomeruli were normally perfused. Under these experimental conditions such is apparently the case.

SUMMARY

1. The number of active glomeruli perfused after injection of a fluorescent dye, thioflavin-S, has been determined in 18 normal rats and 41 rats at varying intervals after administration of 10 mg. per kilogram of body weight of mercuric chloride.

2. There was no correlation between counts of actively perfused glomeruli and either the urine volume or the interval of time following injection of the mercury. There was likewise no correlation between the degree of histologic damage to the tubules and the corresponding counts of active glomeruli. The degree of oliguria, on the other hand, correlated best with the degree of tubular damage.

3. It is concluded that in this experimental lesion the maintenance of anuria is not dependent upon a critical reduction in glomerular perfusion and that diuresis may take place without change in such perfusion. The findings suggest that the oliguria is best explained by back-diffusion of glomerular filtrate rather than glomerular ischemia.

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ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY AND METHEMOGLOBIN REDUCTION

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THE in vitro reduction of methemoglobin by erythrocytes in the presence of glucose and methylene blue is believed to be dependent on the generation of reduced triphosphopyridine nucleotide.†[1] It is the purpose of this paper to correlate the erythrocyte glucose-6-phosphate dehydrogenase* with this TPNH dependent reduction of methemoglobin by erythrocytes in vitro in cord blood and in the blood of patients with diminished erythrocyte G-6-PD activity.

MATERIALS AND METHODS

Erythrocytes from 7 patients with known glutathione instability, 40 cord bloods, and 13 normal adults were studied.

Blood was collected, using either heparin or 0.5 per cent EDTA in normal saline as the anticoagulant. All samples were less than 3 hours old and stored at 8° C. until tested. Whole blood was diluted with 0.85 per cent saline to roughly 6 times its volume, centrifuged at 3,000 r.p.m. for 5 minutes, and the supernatant fluid removed. A volume of 0.25 per cent sodium nitrite equal to the volume of packed cells was added to the erythrocytes, and the suspension was gently agitated for one minute or less and diluted to approximately 40 ml. with 0.85 per cent saline. This was immediately centrifuged for 5 minutes at 3,000 r.p.m., the supernatant fluid removed, and the cells washed 6 times with large volumes of 0.85 per cent saline. After the last washing, the supernatant fluid was removed and the cells resuspended in an equal volume of phosphate saline (M/15 phosphate buffer, pH 7.4, diluted 1:1 with 0.85 per cent saline). A 1 ml. aliquot of the erythrocyte suspension was added to 1 ml. of 0.5 per cent glucose in phosphate saline (2.76 mM. of glucose per milliliter) plus 0.01 ml. of 0.1 per cent methylene blue in normal saline, and the methemoglobin concentration was then determined immediately using the method of Evelyn and Malloy.[2] The mixture was allowed to stand at room temperature with hourly aeration by gentle blowing through a pipette, and the methemoglobin concentration redetermined at 180 minutes. As previously reported in the normal subjects, the methemoglobin reduced was proportional to the initial methemoglobin concentration,[3, 4] and results were expressed, therefore, as per cent of zero hour methemoglobin reduced at 180 minutes.

Erythrocyte G-6-PD activity was determined as described previously.[3] To washed cells, from which the supernatant plasma and buffy coat had been removed, was added an

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†The following abbreviations will be used: TPN = triphosphopyridine nucleotide, oxidized form; TPNH = reduced triphosphopyridine nucleotide; G-6-PD-glucose-6-phosphate dehydrogenase.

equal volume of distilled water. The resultant hemolyzate was dialyzed overnight against 2,000 ml. of distilled water at 8° C. and then assayed. The reaction mixture consisted of 15 μM of the sodium salt of glucose-6-phosphate,* 4 μM of TPN,* and hemolyzate in a final dilutation of 0.05 or 0.1 Gm. This was maintained at pH 7.4 with 0.032 M tris buffer in a final volume of 3 ml. The change in optical density at 340 lambda was observed with the Beckman UV spectrophotometer using a hydrogen lamp source and 1 cm. silica cells. Observations were made for 40 to 50 minutes, and 5-minute increments were averaged. Results were expressed as delta optical density (O.D.) per 5 minutes for 0.1 Gm. per cent hemoglobin.

RESULTS

Cord blood erythrocytes from 19 Negro infants, 16 white infants, and 5 in whom the racial identity was unavailable were tested. The erythrocytes of 5 of the 19 Negro infants were deficient in G-6-PD activity with values below the normal range of 0.040 to 0.096 delta O.D. per 5 minutes.[3] Three of the mothers of these 5 infants were tested and were also found to have low erythrocyte G-6-PD values, presumably demonstrating that this enzymatic defect was hereditary rather than transient. None of the 16 white infants tested were deficient in G-6-PD, while one was deficient among the 5 of unidentified race. The mean erythrocyte G-6-PD activity of the Negro infants was 0.070 delta O.D. while that of the white infants was 0.111. Erythrocyte G-6-PD activity of 5 Negro and 7 white infants was above the upper limits for normal adults.

Methemoglobin reduction by erythrocytes of 13 normal adults was over 70 per cent in 24 determinations. All were Caucasian with previously demonstrated normal erythrocyte G-6-PD.

Reduction of methemoglobin by cord blood erythrocytes in the presence of glucose and methylene blue was deficient in 12 of the 40 infants tested. Nine of these infants were Negro. The deficiency was severe in 8 infants in whom less than 60 per cent of the zero hour methemoglobin was reduced after 180 minutes' incubation. Five of these latter infants were Negroes with low erythrocyte G-6-PD values (Fig. 1), while 3 had normal or increased activity of this enzyme. Only one infant of the series exhibited a low G-6-PD activity associated with normal ability to reduce methemoglobin in the presence of glucose and methylene blue. Conversely, in 7 of 9 samples in which the G-6-PD value was below 0.050 delta O.D. per 5 minutes, methemoglobin reduction was also diminished (Fig. 1). Above that value, methemoglobin reduction was normal in 26 of 31 samples. There were 2 infants with markedly deficient reduction of methemoglobin but normal or high G-6-PD values.

Seven Negro children and adults with known glutathione instability were also tested. The erythrocyte G-6-PD values were low in all, and in all but one (R. L.) there was markedly deficient reduction of methemoglobin in the presence of glucose and methylene blue (Table I and Fig. 2). The erythrocyte G-6-PD level of this seventh case was very low, but reduction of methemoglobin was complete at 180 minutes. Repeated studies of several of

*Obtained from the Sigma Chemical Company, St. Louis, Mo.

the other subjects were consistently low with the exception of Patient D. A., whose methemoglobin reduction was deficient on only 2 of 3 occasions (Table I).

TABLE I. METHEMOGLOBIN REDUCTION IN VITRO IN THE PRESENCE OF GLUCOSE AND METHYLENE BLUE BY ERYTHROCYTES OF 7 GLUTATHIONE UNSTABLE SUBJECTS COMPARED WITH THE ERYTHROCYTE G-6-PD VALUES

PATIENT	DATE	METHEMOGLOBIN AT ZERO HOUR (GM. %)	METHEMOGLOBIN REDUCED AT 180 MIN.		G-6-PD DELTA O.D. PER 5 MINUTES
			GM./10 GM.*	% OF 0 HOUR	
1. D. A.	12/ 2/57	9.4	1.1	11.7	–
D. A.	1/14/58	5.5	4.4	80.0	–
D. A.	11/ 2/58	10.0	1.0	10.0	0.003
2. L. S.	2/ 3/58	10.6	5.0	47.0	0.031
3. J. S.	2/ 3/58	8.2	5.2	63.0	0.025
4. E. A.	2/ 4/58	8.0	2.1	25.0	0.015
5. F. M.	6/ 2/58	9.2	1.3	14.1	0.004
6. I. J.	11/10/58	8.3	1.5	18.2	0.026
7. R. L.	2/11/58	4.4	4.4	100.0	0.001

*Grams of methemoglobin per 10 grams total hemoglobin.

DISCUSSION

The in vitro reduction of methemoglobin by erythrocytes in the presence of glucose and methylene blue is believed to be dependent on the generation of TPNH.[1] In erythrocyte glycolysis there are only two reactions producing TPNH, both steps in the hexose monophosphate shunt.[5] The first of these is the generation of TPNH upon the oxidation of glucose-6-phosphate to 6-phosphogluconate in the presence of G-6-PD:

$$\text{Glucose-6-Phosphate} + \text{TPN} \xrightarrow{\text{G-6-PD}} \text{6-Phosphogluconate} + \text{TPNH} \quad (1)$$

The second reaction, dependent on the normal functioning of the first, generates TPNH upon the oxidation of 6-phosphogluconate in the presence of 6-phosphogluconic dehydrogenase (6-PGD):

$$\text{6-Phosphogluconate} + \text{TPN} \xrightarrow{\text{6-PGD}} \text{Ribose-5-Phosphate} + \text{TPNH} \quad (2)$$

It is now well recognized that there is an inherited inborn error of erythrocyte metabolism resulting in a deficiency of TPNH generation in the presence of glucose-6-phosphate and presumably due to decreased G-6-PD.[3, 6-8] That such individuals have decreased ability to reduce methemoglobin in the presence of glucose and methylene blue has been suggested previously[3] and our present studies confirm that this is usually the case. Thus, the generation of TPNH enzymatically by G-6-PD may be a rate-limiting step in the reduction of methemoglobin in the presence of glucose and methylene blue. An occasional individual with severe deficiency of G-6-PD, however, can apparently reduce normal amounts of methemoglobin despite deficient TPNH generation. This finding is unexplained, but it is unlikely that it is due to variations in technique, since repeated enzyme assays of the individuals involved yielded similar results.

In cord blood, a similar situation prevails. In blood samples in which the values for G-6-PD were diminished or low normal, there was an associated

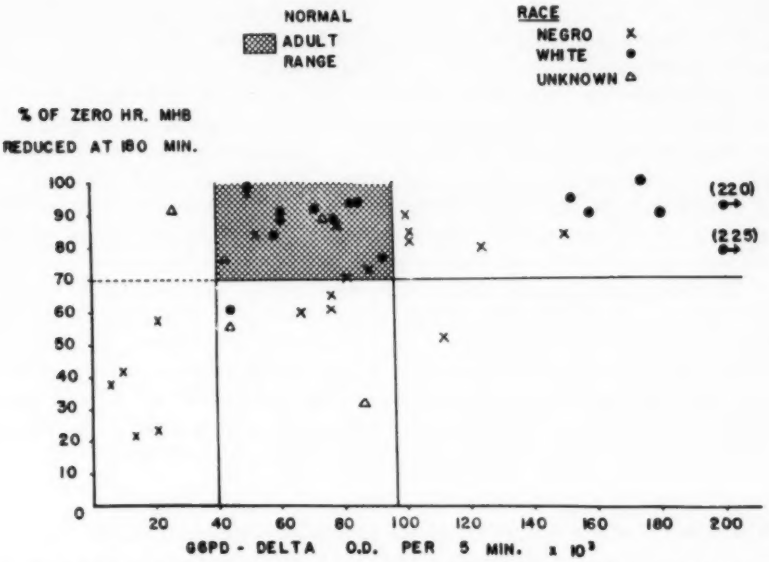

Fig. 1.—Cord blood erythrocyte G-6-PD activity plotted against ability of erythrocytes to reduce methemoglobin in vitro in the presence of glucose and methylene blue. The horizontal line represents the lower limit of normal for reduction of methemoglobin while the two vertical lines represent the lower and upper limits of the normal adult range for G-6-PD activity.

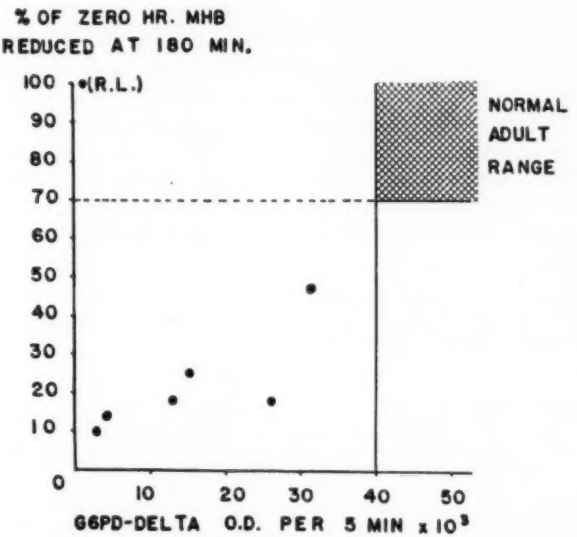

Fig. 2.—Erythrocyte G-6-PD activity of 7 subjects with known glutathione instability plotted against the ability of the erythrocytes to reduce methemoglobin in vitro in the presence of glucose and methylene blue. The horizontal line represents the lower limit of normal for reduction of methemoglobin while the vertical line represents the lower limit of normal for G-6-PD activity.

deficiency of the methemoglobin reduction system. In samples in which the range of activity was higher, methemoglobin reduction was usually normal. In a smaller series previously reported, deficient methemoglobin reduction was less frequent and was thought to be related to decreased G-6-PD,[4] but it is evident that diminished methemoglobin reduction may occasionally occur in the presence of adequate TPNH generation. Infrequently, adequate methemoglobin reduction in the presence of decreased G-6-PD was also noted.

It has been demonstrated by Lonn and Motulsky[9] that the enzyme isolated by Huennekens, methemoglobin reductase,[10, 11] is deficient in cord blood. It is evident that either TPNH generation or other enzymatic steps in the reduction of methemoglobin may be deficient in cord blood. A defect of any or all of the steps may then limit the amount of methemoglobin reduced. Such defects may be transient and related to immaturity of enzyme systems or permanent and determined by heredity. In the blood of older subjects without deficiency in methemoglobin reductase the generation of TPNH may determine the rate of methemoglobin reduction, and our findings in the blood of patients with G-6-PD deficiency are consistent with this postulate.

SUMMARY

A correlative study was made of the ability of cord blood and of G-6-PD deficient erythrocytes to generate TPNH and to reduce methemoglobin in vitro in the presence of glucose and methylene blue. Deficient methemoglobin reduction was found to be associated frequently with decreased G-6-PD activity in cord blood and in the blood of patients with congenital G-6-PD deficiency. In cord bloods with G-6-PD activity greater than 0.050 delta O.D. per 5 minutes, methemoglobin reduction was usually within normal limits, although deficient reduction occasionally occurred in the presence of normal TPNH generation. Thus, it would appear that generation of TPNH may be a rate-limiting step for the reduction of methemoglobin in a glucose and methylene blue system. Since in both cord blood samples and samples from patients with G-6-PD deficient red cells there was an occasional lack of correlation between these two activities, it is evident that other factors may play a part in determining the rate of methemoglobin reduction.

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ACONITASE IN HUMAN BLOOD

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BLOOD is the tissue that may be "biopsied" most readily for the clinical determination of the activity of enzymes in living cells.

In the course of studies of iron enzymes in patients with iron deficiency, we thought it desirable to obtain measurements of the enzyme aconitase, which catalyzes the equilibrium between citric, isocitric, and aconitic acids.[1] It is the purpose of this communication to describe a method for the semiquantitative determination of the activity of this enzyme in human blood cells and to present the results of assays carried out on normal subjects and those with a variety of clinical disorders.

METHODS

A. Preparation of Enzyme Solution.—

1. White cell-platelet layer: Twenty to 40 ml. of blood was obtained by venipuncture. Heparin was used as the anticoagulant. One-fifth volume of a solution of polyvinylpyrrolidone† was mixed with the blood in a test tube, and the red cells were allowed to settle for 1 hour at 37° C. in the tube positioned at a 45-degree angle. The supernatant plasma layer was then pipetted off, its volume was measured, and a white count and chamber differential count were performed.

The white cell-rich layer was centrifuged at 100 g for 10 minutes. The supernatant was discarded, and a few milliliters of phosphate-acetate buffer‡ were added. The white cells were stirred into as even a suspension as possible, and the suspension was recentrifuged at 90 g. Clumps of the sedimented white cells were broken up in a Potter-Elvejhem type tissue homogenizer, and the homogenized white-cell suspension was brought to a total volume of 7.5 ml. with buffer. This procedure does not release appreciable amounts of enzyme from the cells; the white cells were disrupted by freezing and thawing twice, alternatively immersing them in a Dry Ice alcohol bath and a 37° C. water bath. Additional freezings and thawings did not increase enzymatic activity.

2. Platelet layer: A platelet-containing layer with relatively few white cells was prepared by subjecting a white cell-platelet suspension to slow centrifugation (approximately 20 g) for 3 minutes. A white cell count and direct platelet count was carried out on the supernatant fluid, and an enzyme solution was prepared from the fluid as in paragraph 1 above, except that it was brought to a volume of only 4.0 or 4.5 ml.

3. Red cell layer: After removal of the white cell-platelet layer, the PVP sedimented red cells were washed in 0.9 per cent NaCl solution and packed by centrifugation.

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†PVP-Macrose, Schenley Laboratories, Inc., New York, N. Y.

‡Prepared by dissolving 2 Gm. sodium acetate in water, adding 1.42 ml. of 85 per cent H_3PO_4, adjusting pH to 7.40 with 5 per cent NaOH, and bringing the volume to 1,000 ml. with water.

Any remaining buffy coat was removed. A white cell count was performed. The enzyme solution was prepared by freezing and thawing two times in a Dry Ice alcohol bath.

4. Plasma: Heparinized plasma from which all cellular elements had been removed by centrifugation was used without further treatment.

B. Preparation of Substrate.—*Cis*-aconitic anhydride was obtained from the Sigma Chemical Company. A weighed portion of *cis*-aconitic anhydride was dissolved in a small quantity of water and allowed to remain in aqueous solution at room temperature for 1 hour. At the end of this time, 0.1 ml. of 2 N NaOH was added per 16.6 mg. of *cis*-aconitic acid and sufficient phosphate acetate buffer was added to give a final concentration of 7.8 mg. anhydride per milliliter. The substrate solution must be prepared on the day of assay.

C. Assay of Aconitase Activity.—Each enzyme solution, except the one from platelets, was assayed at 2 and 4 ml. levels. The platelet-enzyme solution was assayed only at the 4 ml. level. The enzyme solution was brought to a total volume of 4.5 ml. by the addition of phosphate-acetate buffer. The reaction was started by the addition of the substrate (aconitate) and was stopped after 15 minutes' incubation at 37° C. by adding 2 ml. of 50 per cent trichloroacetic acid (TCA). The reaction tube was centrifuged, and 5 ml. of the supernatant solution was pipetted into a tube graduated at 10 and 25 ml. for citrate analysis. The citrate is stable in TCA solution and may be determined at a later time. Citrate determinations carried out on blanks with substrate without blood cells and on blanks with blood cells but without substrate with and without incubation were consistently found to be negative in preliminary studies and were not carried out in the assays reported here, except in the case of studies with plasma, where plasma citrate blanks were determined.

D. Citrate Determination.—Determinations of citrate were carried out by a minor modification of a method described in detail elsewhere.[2] Five-tenths milliliter of 50 per cent (v/v) H_2SO_4, 0.2 ml. 1 M KBr, and 1 ml. saturated $KMnO_4$ solution were added to the TCA filtrate. After standing at room temperature for 5 to 20 minutes, the tubes were placed in an ice bath and the excess permanganate was removed by the dropwise addition of 1 per cent H_2O_2. The volume was brought to the 10 ml. mark with H_2O and to the 25 ml. mark with acid-washed n-heptane. After shaking for 5 minutes, 10 ml. of the heptane layer was added to 10 ml. of a reagent composed of 4 per cent thiourea and 2 per cent Na borate in a Coleman junior 25 mm. cuvette. After shaking for 5 minutes, the mixture was centrifuged lightly and read at 445 and 650 mμ against a reagent blank. The reading at 650 mμ was subtracted from the reading at 445 mμ, and the citric acid concentration was read from a curve constructed from 50, 100, and 200 μg citric acid standards.

E. Calculation of Results.—The citric acid content of the 5 ml. TCA filtrate is multiplied by 1.4 to give the citric acid formed in the 5 ml. reaction mixture. We have defined one unit of activity as that amount of enzyme that will convert 0.85×10^{-7} moles of aconitate to citrate per minute in the 5 ml. assay system. Thus, 1 unit of enzyme will result in the appearance of 245 μg (12.75×10^{-7} M) of citrate in the reaction mixture. Using rat kidney (for which we originally developed this assay system) as source of enzyme, the amount of citrate formed was found to have an approximately linear relationship to the volume of enzyme solution added over a range of 0.15 to 2.0 units of enzyme. An approximately linear relationship between volume of enzyme solution and activity has been observed also in the blood cell preparations studied.

Enzyme activity in red cells has been corrected for entrapped white cells and is expressed as units per milliliter of red cells. This value closely approximates units per 10^{10} red blood cells. White cell-platelet layer activity has been expressed as units per 10^{10} white cells because most of the activity found was in the leukocytes. Platelet activity was estimated after correcting for the activity in the remaining white cells. Because red cell activity was so low and because the method used causes red cell lysis, no correction for red cell activity was necessary.

RESULTS

A. Plasma Activity.—No appreciable enzyme activity could be found in the plasma of 6 normal subjects, in the plasma of one subject with active cirrhosis of the liver, or in the plasma of one subject with acute viral hepatitis and one subject with acute myocardial infarction.

B. Red Blood Cell Activity.—The red blood cells of 5 normal subjects were calculated to contain 0.025, 0.029, 0.031, 0.038, and 0.038 unit of activity per milliliter. Red cells of the sixth subject were estimated to contain no activity after correction for entrapped white cell activity had been made. Estimations of red cell aconitase were also carried out on one subject with a reticulocyte count elevated to 30 per cent during treatment of an iron deficiency anemia. The activity of the red cells of this patient was 0.81 unit per milliliter.

C. Leukocyte-Platelet Activity.—The results of aconitase assay of the white cell-platelet layer of 6 normal individuals and 11 patients with a variety of clinical disorders are given in Table I. It is apparent that the activity per 10^{10} white blood cells is relatively constant in normal subjects. It is not technically feasible to study separately the activity of granulocytes and lymphocytes, but the data do not appear to disclose an appreciable correlation between the differential formula and enzyme activity; in the normal group, the samples with the lowest and the highest aconitase activity had almost the same percentage of mononuclear cells. It seeems likely, therefore, that there is not very great difference between the aconitase activity of these two types of cells. Unusually low values of aconitase were found in one patient with acute leukemia and in one with multiple myeloma. The white cell aconitase activity of two patients with iron deficiency anemia was normal.

D. Platelet Activity.—The results of determinations of platelet activity varied widely, probably because of inaccuracies in the enumeration of platelets and because platelet preparations were contaminated invariably with leukocytes. The activity per 10^{10} platelets of 5 hematologically normal subjects was calculated to be 0.4, 0.8, 1.1, 2.7, and 5.2 units respectively. A subject with a lymphoma had a platelet activity of 1.3 units per 10^{10} platelets, and two patients with polycythemia rubra vera had platelet activities of 0.42 and 2.73 units per 10^{10} platelets.

DISCUSSION

To our knowledge, there are no published reports of the aconitase activity of human blood. In point of fact, the only reported studies of which we are are aware of aconitase activity in blood cells of any species are assays carried out on rabbit reticulocytes by Rubinstein and associates.[3] These workers found enzyme activity to be present. However, no white cell assays were made, and no correction was made for entrapped white cells.

Our investigation demonstrates that the activity of the enzyme aconitase in human blood cells can be measured. Because of the well-known difficulty in preparing highly purified suspensions of a single blood cell type, the results of these studies should not be considered to be more than semiquantitative estimates. Nonetheless, remarkably uniform results are obtained in normal

subjects. Variations in the diseases studied, with the possible exception of myeloma and acute lymphocytic leukemia, are unimpressive. Activity was found to be very low in red cells when compared with white cells and platelets—a finding consistent with the virtual absence of the citric acid cycle in mature erythrocytes.[4]

TABLE I. WHITE CELL-PLATELET LAYER ACONITASE ACTIVITY

DIAGNOSIS	SEX	CHAMBER DIFFERENTIAL		PERIPHERAL BLOOD PLATELETS / LEUKOCYTES	ACONITASE ACTIVITY (UNITS PER 10^{10} W.B.C.)
		PML*	MONONUCLEAR LEUKOCYTES		
Normal	M	52	48	202,400 / 10,450	104
Normal	M	50	50	---	88
Normal	M	52	48	---	66
Normal	F	63	37	---	150
Normal	F	57	43	---	107
Normal	F	61	39	---	65
Chronic leg ulcer	M	65	35	176,000 / 8,450	128
Infectious mononucleosis	F	34	66	157,300 / 6,550	45
Hypopituitarism	M	62	38	166,100 / 10,950	71
Iron deficiency anemia	F	73	27	200,200 / 7,000	106
Iron deficiency anemia	F	70	30	---	94
Polycythemia rubra vera	M	89	11	800,000 / 23,500	162
Chronic lymphatic leukemia	M	3	97	53,000 / 103,700	87
Chronic myelogenous leukemia	M	55	45	152,400 / 89,000	104
Acute lymphocytic leukemia	F	5	95	117,700 / 226,000	21
Acute monocytic leukemia	F	8	92	66,000 / 26,000	242
Plasma cell myeloma	M	63	37	166,000 / 3,400	35

*Polymorphonuclear leukocytes.

SUMMARY

A technique for measuring the activity of the enzyme aconitase in human blood has been described. Normal plasma was found to be devoid of enzymatic activity, and only small amounts of activity were observed in red blood cells from normal individuals. A patient with marked reticulocytosis had much higher red cell aconitase levels. The bulk of activity was present in white cells

and platelets. Estimates of activity must be considered semiquantitative because of the difficulty in obtaining pure cell suspensions. The following average normal values are given, however, as a rough guide: erythrocytes, 0.026 unit per milliliter; leukocytes (not corrected for platelet contamination), 98 units 10^{10} cells; and platelets (corrected for leukocyte contamination), 2.0 units 10^{10} platelets.

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LABORATORY METHODS

THE DETERMINATION OF SULFISOXAZOLE AND SULFADIMETHOXINE IN BLOOD AND PLASMA

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TWO new sulfonamides (2,4-dimethoxy-6-sulfanilamido-1,3-diazine [sulfadimethoxine] and 3,4-dimethyl-5-sulfanilamidoisoxazole [sulfisoxazole])† have recently been developed as bacteriostatic agents. In contrast to sulfisoxazole, which is rapidly excreted in urine,[1] blood levels of sulfadimethoxine may be maintained for 24 hours following a single oral dose.

During the course of a series of clinical trials designed to establish a relationship between whole blood and serum levels with clinical effectiveness, it became apparent that sulfadimethoxine and sulfisoxazole were markedly bound to blood and serum proteins precipitated with trichloroacetic acid. The degree of binding, considerably higher than other commonly used sulfonamides, suggested the need for correction factors to compensate for the drug adsorbed to the precipitate. The results of this study form the basis for this report.

MATERIALS AND METHODS

A. Procedure.—The concentration of various sulfonamides in trichloroacetic acid extracts of blood and serum was determined by the procedure of Bratton and Marshall.[2] The following sulfonamides were used:

A. Sulfanilamide (Merck).
B. Sulfanilamide (Eastman Kodak).
C. Sulfadiazine (Sharp & Dohme).
D. p-Aminobenzoic acid (General Biochemicals).
E. Sulfathiazole (Merck).
F. Sulfapyridine (Dagenan).
G. Sulfisoxazole (Hoffmann-La Roche).
H. Sulfadimethoxine (Hoffmann-La Roche).

B. In Vitro Studies.—

1. Rate of adsorption: The various drugs, added to whole blood and serum, were extracted with trichloroacetic acid at a final blood or serum dilution of 1:20. With the exception of p-aminobenzoic acid which was added at a level of 8 mg. per 100 ml. of blood or

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†Sulfadimethoxine is commercially available as Madribon and sulfisoxazole as Gantrisin, Hoffmann-La Roche, Inc., Nutley, N. J.

serum, all other drugs were present at a level of 12 mg. per 100 ml. The mixtures were refrigerated at 4° C. and mixed 2 or 3 times daily during a 2-week period. At intervals, a portion of the mixtures were filtered and the concentration of drug in the filtrate was determined. The difference between the amount found in the filtrate and the amount originally added represents the amount adsorbed to the denatured proteins. Identical solutions, in the absence of blood proteins, were carried through the procedure and were found to be perfectly stable.

2. Effect of protein concentration on adsorption: Hemoglobin solutions were prepared from human erythrocytes washed 3 times with saline. The washed cells were hemolyzed with an equal quantity of distilled water and a portion of the hemolyzate was centrifuged to remove red cell ghosts and stromal elements. The other portion was used without further treatment. Varying amounts of the hemoglobin solutions were added to flasks containing sulfadimethoxine at a final concentration of 6 mg. per liter. Following the addition of trichloroacetic acid, the mixtures were filtered after either 10 minutes or 7 days' refrigeration and the filtrates were analyzed for residual sulfonamide. Human serum, filtered after 10 minutes, replaced the hemoglobin solutions for comparative purposes.

3. Type of adsorption: The adsorption of sulfadimethoxine to hemoglobin precipitated by trichloroacetic acid was further studied by adding graded amounts of the drug to flasks containing hemoglobin solutions at a final protein concentration of 6.5 Gm. per liter. Following the addition of trichloroacetic acid, the mixtures were filtered after 10 minutes or refrigeration for 7 days and the filtrates were analyzed for residual sulfonamide.

4. Determination of correction factors: Recovery studies were performed by adding appropriate amounts of various sulfonamides to whole blood and serum followed by extraction with trichloroacetic acid. The final dilution of blood and serum was 1:20 and filtration was performed after 10 minutes. The concentration of sulfonamide in the filtrates was determined using the appropriate sulfonamide as the standard. The blood and serum were obtained, for the most part, from the routine laboratory. Blood and serum from healthy individuals were used in some instances. In general, the hematocrits ranged between 33 to 49 per cent.

C. In Vivo Studies.—

1. Blood levels of sulfadimethoxine: Single oral doses were given to children with various infectious diseases. The children ranged in age from 1 to 12 years, and the drug was administered at a level of 30 mg. per kilogram of body weight. At suitable intervals, blood samples were obtained and the concentration in blood and plasma, corrected for adsorption to the precipitate and for the hematocrit, was used to determine the concentration and distribution of the drug.

2. Blood levels of sulfisoxazole: Blood samples from adult patients with various infectious diseases receiving oral sulfisoxazole at various therapeutic and prophylactic dosages were analyzed for free sulfonamide. The concentration and distribution of drug in whole blood and plasma were corrected for adsorption to the precipitate and for the hematocrit.

RESULTS AND DISCUSSION

In whole blood, the drugs are initially bound to denatured protein in varying degrees when the mixtures were allowed to stand for 10 minutes before they were filtered (Fig. 1). Thus, sulfanilamide (A) is not bound to any significant degree while 20 per cent of sulfisoxazole (G) and 22 per cent of sulfadimethoxine (H) are bound to the denatured protein. The adsorption process continues, however, and finally reaches an equilibrium value. When equilibrium is reached, as much as 40 to 50 per cent of sulfisoxazole and sulfadimethoxine may be removed from solution as compared to 25 per cent adsorption for sulfapyridine (F).

Sulfonamides A to F are bound to a lesser extent to denatured serum proteins, but 15 to 20 per cent of sulfisoxazole or sulfadimethoxine may be bound. It is interesting to note that in serum these two sulfonamides do not reach equilibrium for 2 to 4 days while the other sulfonamides reach adsorption equilibrium almost immediately.

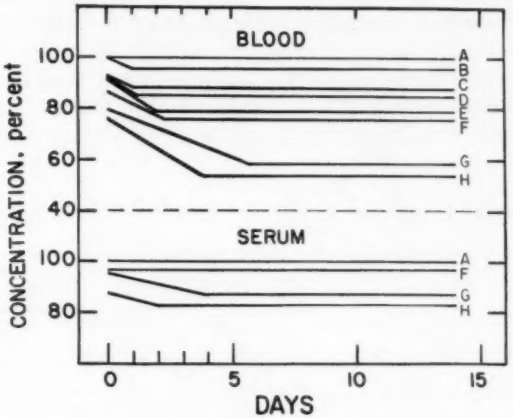

Fig. 1.—The filtrate concentration of various sulfonamides in the presence of trichloroacetic acid precipitates of whole blood and serum as a function of time. The letters represent the various drugs used (see text under Materials and Methods).

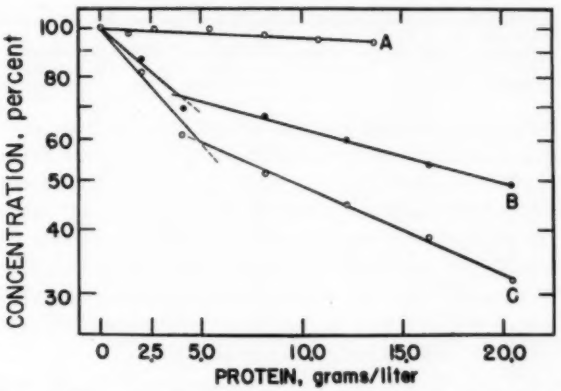

Fig. 2.—The filtrate concentration of sulfadimethoxine in the presence of hemoglobin and serum proteins precipitated with trichloroacetic acid. Key: A = serum filtered after 10 minutes; B = hemoglobin filtered after 10 minutes; C = hemoglobin filtered after 7 days' refrigeration.

With increasing hemoglobin concentration, two curves with different slopes became apparent, thus indicating the presence of two sites for adsorption (Fig. 2). Although the amount of drug adsorbed on the denatured protein increases with time, the same binding sites are present for solutions filtered after 10 minutes (Curve B), or filtered at conditions of equilibrium after 7

days (Curve C). Similar curves were obtained with centrifuged stroma-free hemolyzates, thus indicating that stromal elements and erythrocyte ghosts adsorb little, if any, of the drug. In serum, the drug concentration decreased in a regular fashion with increasing serum protein concentration (Curve A), thus indicating the presence of one weak binding site. The relationship between the amount of sulfadimethoxine adsorbed per unit of hemoglobin is adequately described by a Freundlich adsorption isotherm under the experimental conditions used, thus indicating an equilibrium process (Fig. 3).

As shown in Table I, the recovery of more commonly used sulfonamides, including p-aminobenzoic acid, ranges between 87.5 to 92.9 per cent. In contrast to these values, sulfisoxazole and sulfadimethoxine are recovered to only 73.2 and 74.3 per cent, respectively.

TABLE I. CORRECTION FACTORS FOR VARIOUS SULFONAMIDES ADDED TO WHOLE BLOOD AND SERUM

DRUG	WHOLE BLOOD		SERUM	
	PER CENT RECOVERY	CORRECTION FACTOR	PER CENT RECOVERY	CORRECTION FACTOR
Sulfisoxazole (Hoffmann-La Roche)	73.2 ± 3.70*	1.40 ± 0.08*	85.3 ± 1.44*	1.17 ± 0.02*
Sulfadimethoxine (Hoffmann-La Roche)	74.3 ± 4.44	1.37 ± 0.09	87.5 ± 1.39	1.14 ± 0.02
Sulfanilamide (Merck)	91.7 ± 1.58	1.09 ± 0.02	96.0 ± 0.39	1.04 ± 0.01
Sulfanilamide (Eastman Kodak)	92.9 ± 4.11	1.08 ± 0.05	96.1 ± 1.90	1.04 ± 0.02
Sulfathiazole (Merck)	87.5 ± 2.78	1.15 ± 0.04	92.5 ± 3.38	1.08 ± 0.04
Sulfapyridine (Dagenan)	90.0 ± 2.11	1.11 ± 0.03	95.0 ± 0.57	1.05 ± 0.01
Sulfadiazine (Sharp & Dohme)	91.3 ± 1.19	1.10 ± 0.02	93.8 ± 2.46	1.07 ± 0.03
p-Aminobenzoic acid (General Biochemicals)	89.1 ± 2.39	1.12 ± 0.03	93.8 ± 0.88	1.07 ± 0.01

All drugs were added to blood and serum equivalent to 12 mg. per cent; p-aminobenzoic acid was equivalent to 8 mg. per cent.

All values represent average values obtained for 3 to 5 different samples of blood or serum.

*Standard deviation.

The above data indicate that analysis of sulfadimethoxine and sulfisoxazole in blood and serum requires the application of correction factors to correct for the adsorption of these drugs to blood proteins. Since the adsorption of these drugs is primarily to hemoglobin (Figs. 2 and 3), the values obtained will vary with the hemoglobin concentration and the correction factors can only be considered as approximations. On the other hand, the adsorption of these drugs is considerably less for serum proteins than for hemoglobin, and the correction factors will not vary as much with the changes in protein concentration encountered in pathologic conditions.

Following a single oral dose of sulfadimethoxine, blood and serum levels reach and maintain a maximum concentration between 6 and 24 hours (Fig. 4). Thereafter, the concentration decreases slowly. After 72 hours following the single dose, the plasma still contains 5 mg. per 100 ml. During the entire 3-day experimental period, serum was found to contain between 80 and 90 per cent of the total blood concentration. In 5 adult patients receiving oral sulfisoxazole, the serum contained 85 per cent of the total blood concentration at high or low blood levels (Table II). It would appear, therefore, that the use of serum is preferable to whole blood for the estimation of blood levels of these two sulfonamides.

The application of the Bratton-Marshall procedure for the estimation of sulfonamides requires a correction factor for the quantity of drug which is bound to proteins denatured by trichloroacetic acid. For clinical purposes, adsorption of less than 10 per cent has usually been ignored. Thus, Fister[3] gives no correction factors for 7 sulfonamides in clinical use but lists those which may be performed in whole blood and those in which serum or plasma

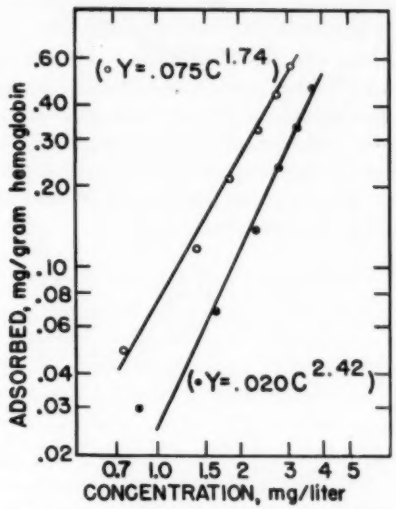

Fig. 3.—The relationship between concentration of sulfadimethoxine and the amount bound to trichloroacetic acid-precipitated hemoglobin. The lines were fitted by the method of least squares. Key: open circles (○) = hemoglobin filtered after 7 days' refrigeration; closed circles (●) = hemoglobin filtered after 10 minutes.

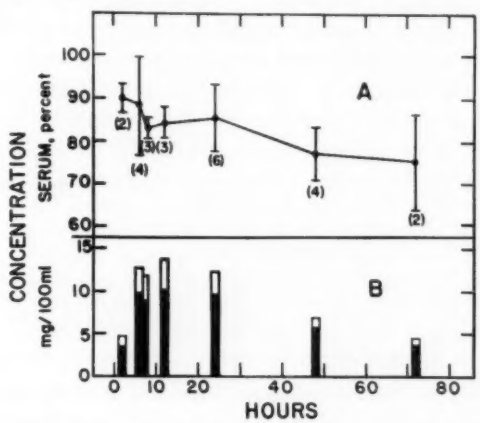

Fig. 4.—The blood and serum concentrations and distribution following a single oral dose of sulfadimethoxine. *A*, The per cent of the total blood concentration present in serum versus time. The vertical lines represent ± 1 standard deviation and the numbers in parentheses represent the number of samples. *B*, The concentration of sulfadimethoxine in whole blood and serum. The total bar represents whole blood, and the solid portion of the bar represents serum concentration.

is preferred. Hepler[4] prefers serum for all sulfonamide determinations, while Hawk and associates[5] use whole blood for these determinations. These authors completely disregard the fact that most sulfonamides, with the exception of sulfanilamide, sulfacetimide, and sulfaguanidine, are predominantly present in the plasma. Although Hepler does not give correction factors, Hawk and associates suggest that correction factors are not necessary for low concentrations (5 mg. per cent) of sulfanilamide, sulfaguanidine, sulfapyridine, or sulfathiazole, but apply a correction for sulfapyridine when present in amounts greater than 5 mg. per cent. On the other hand, Scudi and Robinson[6] perform the analyses in dilutions of 1:50 rather than 1:20 in order to avoid correction for adsorption. The larger dilutions decrease the sensitivity of the procedure to some extent. The more concentrated solutions from 1:20 dilutions are more applicable to modification of the Bratton-Marshall procedure for microanalysis; thus, under these conditions, correction factors should be applied in order to obtain correct sulfonamide determinations.

TABLE II. DISTRIBUTION OF SULFISOXAZOLE IN BLOOD AND PLASMA FOLLOWING ORAL ADMINISTRATION

SAMPLE	HCT	WHOLE BLOOD (MG./100 ML.)	SERUM (MG./100 ML.)	PER CENT IN SERUM
A	38	18.5	24.8	83.0
B	42	1.83	2.69	85.3
C	43	4.94	7.90	91.2
D	42	8.17	12.61	89.6
E	39	2.40	2.92	74.1
		Average		84.6

SUMMARY

The sulfonamides, sulfadimethoxine and sulfisoxazole, are predominantly present in serum, suggesting that serum is to be preferred over whole blood for analytic purposes. Both drugs are adsorbed to precipitated blood proteins, but the adsorption to acid-precipitated serum proteins is much less than the adsorption to denatured hemoglobin. The adsorption of these drugs to serum proteins requires the application of correction factors in order to obtain accurate, clinically useful determinations.

We are grateful to Dr. Edward F. Townsend for supplying many samples of blood from his patients and to Dr. L. R. Hines, Jr., Hoffmann-La Roche, Inc., for supplies and financial assistance.

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A TECHNIQUE FOR DETERMINING WHOLE BLOOD CLOTTING TIMES IN PLASTIC TUBES

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MANY methods have been used to evaluate the over-all coagulability of the blood. Of these perhaps the most extensively studied is some variant of the whole blood clotting time in either glass or non-wetting containers. Tests of this type have proved most useful in detecting gross clotting deficiencies involving extreme depression of certain coagulation components, or the presence of anticoagulants. However, this group of tests is subject to serious limitations primarily based on the consideration that they fail to reflect the corresponding coagulability of the blood in vivo. Even when extreme precautions are taken to prevent contamination with tissue coagulants, the clotting time of human blood in glass is typically between 8 and 20 minutes. Although the use of siliconized glassware has been reported to give clotting times up to 2 hours,[1] more often values between 18 and 35 minutes are obtained.[2, 3] This rapid clotting of blood in vitro is in sharp contrast to the long coagulation time of blood in a doubly ligated vein segment, in which the blood may remain fluid for several hours or more.[4] Evidently the manipulation of the blood required in the in vitro tests elicits an extreme degree of clotting activation which may result in overwhelming a minor deficiency on one hand, and masking increased coagulability on the other. Factors which may be responsible for this activation include the trauma incident to transferring blood from one container to another, the presence of an air-blood interface,[5] exposure to a wetting or absorbing surface, and agitation of blood necessary for determining clotting end points. The present technique was developed in an attempt to provide an in vitro method minimizing these factors and eliminate some variables not encountered in in vivo conditions. The principle of the method consists in determining clotting of blood held in plastic tubes.

METHODS AND MATERIALS

In studies on human subjects blood specimens were collected in regular blood donor sets.* These sets consist of plastic tubing 90 cm. in length and 3 mm. in inside diameter, with 15 gauge silicone-coated needles at each end. The subjects were hospital personnel of both sexes between the ages of 19 and 60 years in whom there was no evidence of disease. The test was performed as follows. The needle of the donor set designed for insertion into

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*Saftidonor, Cutter Laboratories, Berkeley, Calif.

the blood bottle was cut off. The donor needle was inserted into a suitable vein (usually the antecubital) by clean venepuncture. Unsatisfactory results were invariably obtained if there was difficulty in entering the vein, or if blood flow was intermittent or in a slow dropwise fashion. The tube was allowed to fill by pressure flow, about 5 ml. of blood was allowed to run out, and the tube then was clamped quickly at both ends with hemostats. The needle was withdrawn and cut off, and the tubing was laid flat and kept at room temperature (20° to 22° C.). At 10 minute intervals, aliquots of blood were inspected for the presence of clotting by snipping off segments of the tubing. This was accomplished by applying a third hemostat about 1 cm. from either end hemostat, and cutting with scissors so that the tubing remained doubly clamped, but a small segment had been removed. This segment was opened by removing the hemostat and rinsed to and fro in a Petri dish of isotonic saline placed on a white background. The end point was taken as the time when tiny strands of fibrin were visible either floating free in the saline or adhering to the tubing. If there was doubt, an additional segment of tubing was promptly cut and studied as before.

The technique was adapted for use in rabbits. These were white, male animals of the New Zealand strain, weighing about 3 kilograms. The animals were anesthetized with intravenous Nembutal supplemented with ether as necessary. The right jugular vein was exposed by dissection, its distal tributaries were ligated, the vein was cut open, and a No. 14 trochar was inserted. Lengths of plastic tubing (B-D VX-044) measuring about 30 cm. were cut, and these were slipped over an 18 gauge needle attached to a 10 ml. syringe containing isotonic saline. This tubing was marked at a point measuring the distance from its free end equal to that from the animal's maximal cardiac impulse on the right to the mouth of the trochar. The tubing was filled with saline from the syringe, and clamped with a hemostat next to the needle. It was then inserted into the jugular vein through the trochar and threaded through until the mark on the tubing was at the mouth of the trochar. This manipulation usually placed the tip of the tube in the right atrium, as determined by subsequent postmortem dissection. The hemostat was then removed from the tubing, and the tubing was flushed with about 2 ml. of saline from the syringe. The syringe was then used gently to aspirate blood from the heart, and this was continued until about 2 ml. of blood had entered the syringe, thus displacing all saline from the tubing. The tube was doubly clamped as above, it was withdrawn from the trochar, and the stylet of the trochar was replaced. This procedure made it possible to perform repeated determinations of the clotting time in rapid succession by insertion of fresh tubes. The clotting end point was determined as above, except that the tube was flushed with saline by means of a syringe supplied with an 18 gauge needle.

RESULTS

The data obtained from normal human subjects are presented in Fig. 1. It can be seen that the first evidence of fibrin formation most frequently occurred after 2 hours of incubation. Once such clotting started, about 40 additional minutes of incubation was necessary for completion of the process with the formation of a solid clot. Incubation of the tube at 37° C. resulted in shortening of the clotting time by about 30 minutes, as shown when the tube was divided into 2 segments one of which was immersed in a 37° C. water bath, the other maintained at room temperature. When previously mentioned difficulties in venepuncture were encountered the clotting times were considerably shortened; such specimens were discarded. Multiple tests performed on a single individual usually agreed within 20 minutes, as shown in Table I.

In rabbits the onset of fibrin formation occurred in about 1 hour, and repeated determinations in a given animal agreed within about 10 minutes. The shorter clotting time of rabbit blood may represent differences in technique,

since tubes of smaller caliber were found to give somewhat shorter clotting times in human subjects as well. It is also possible that rabbit blood is inherently more coagulable.

TABLE I. PLASTIC TUBE CLOTTING TIMES (IN MINUTES) TAKEN SUCCESSIVELY FROM OPPOSITE ARMS IN HUMAN SUBJECTS

SUBJECT	RIGHT ARM	LEFT ARM
1	90	90
2	90	90
3	100	100
4	100	110
5	160	170
6	180	160

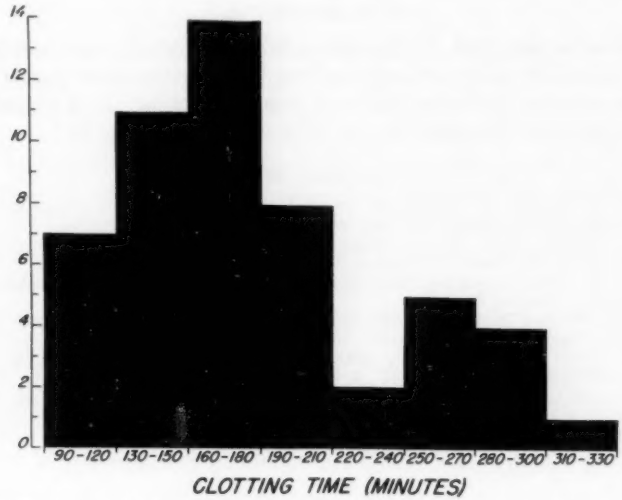

Fig. 1.—Frequency distribution of plastic tube clotting times in normal human subjects.

DISCUSSION

The present technique yields clotting times approaching those encountered by Wessler[4] in doubly ligated vein segments. This prolonged preservation of blood fluidity suggests that coagulability of the blood is being tested in near-physiologic conditions. Since under normal circumstances there is little tendency for the blood to clot in vivo, the technique would not appear to be suitable for the study of coagulation deficiencies. The test was designed primarily for evaluation of "hypercoagulable" states.

At present there is no certain evidence that a generalized increase in blood coagulability occurs in disease, although it would seem likely that such changes might occur. For example, during the course of major surgery or in widespread thromboembolic disease, coagulation-active materials probably enter the general circulation in significant amounts. Since there appears to be little activation of the blood clotting factors in plastic tubes, the method should be sensitive to the presence of an already activated system.

Experimentally there has been unequivocal production of the hypercoagulable state in vivo. Wessler[6] has shown that intravenous administration of serum results in rapid clotting within doubly ligated vein segments remote from the site of injection. In rabbits, following the intravenous injection of rabbit serum, the plastic tube clotting time is markedly shortened; this shows that the method is capable of demonstrating hypercoagulability.

Work is in progress designed to evaluate the plastic tube clotting time under various physiologic and pathologic conditions. At least one conclusion from these data is that there is no demonstrable consistent alteration in normal subjects following meals containing large amounts of lipids derived from butterfat or eggs.

SUMMARY

A method is described for the determination of whole blood clotting times in plastic tubes. By this technique clotting begins after about 2 hours in normal human subjects, and after about an hour in rabbits. It is suggested that the method is useful for studying the hypercoagulable state.

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RAPID SEPARATION AND QUANTITATION OF THREE PHENOLIC ESTROGENS BY PAPER CHROMATOGRAPHY

A Method and Its Evaluations

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SINCE Zaffaroni and associates[1] demonstrated the application of paper chromatography to urinary steroid determinations, investigators have sought a method which could be used to determine estrogen excretion on a clinical basis and which would be accurate and reproducible. Bloch and co-workers[2] showed that by the application of Zaffaroni's technique[1] (the conversion of the estrogens to hydrozones, chromatographing and localizing them with K_2PtI_6) a quantitative method could be adapted for the determination of several urinary steroids. However, they were unable to determine the estrogens quantitatively with the exception of estrone, and, in the determination of this compound, they encountered difficulties which are not easily controlled for accurate quantitation.

Dao[3] did show that it was possible to get good separation of 4 of the 5 known naturally occurring estrogens without changing their chemical nature by the application of paper chromatography. He did, however, use biologic assay as a final method of quantitation. With this method, Dao was able to quantitate as little as 0.03, 0.005, 0.03, and 0.06 μg of estrone, 17-β-estradiol, estriol, and 16-epiestriol, respectively. Huggins and Jensen[4] and Hisaw and associates[5] have shown that there are potential antagonistic actions of the various estrogenic steroids when administered together; it is well known that there may be some potentiation or augmentation of their action in the biologic test animals by the presence of the adrenal glands. Nevertheless, Dao's test for quantitation is quite sensitive and can measure very small amounts of the excreted steroids.

We are of the opinion, however, that Dao's method is too time consuming to be applicable on a routine clinical basis. We feel that a method could be developed which does not depend upon the fluctuating state of the test animals, instead it would be purely physical and chemical and, as such, would have a greater degree of accuracy. This paper presents our results of an investigation of such a method.

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MATERIALS AND METHODS

Estrogens.—17-β-Estradiol, estrone, and estriol were the 3 estrogens used in the experiment.* These were dissolved in redistilled methanol at a concentration of 2 mg. per milliliter. Aliquots of 10, 15, 20, 25, and 30 μg of each estrogen in the mixture were applied to Whatman's filter paper No. 3 and allowed to develop in a sealed glass tank by the descending method.

Solvents.—The developing solvent system employed was n-heptane-methanol. Both reagents were redistilled by a method described by Dao[3] to remove impurities which have been found to be present in the reagent grades and which would interfere with the separation of the steroids. The methanol and heptane were mixed in a 1:1 ratio and allowed to equilibrate in a separatory funnel. This is kept at a temperature of 35° to 37.5° C. The upper and lower layers resulting from the mixture are used as mobile and stationary phases, respectively.

Chromatography.—Whatman's filter paper No. 3 was used for obtaining each separation. This paper consisted of sheets cut to the size of 9.9 by 41 cm. The sheets were prepared by washing in a soxhlet type apparatus with redistilled absolute methanol for 3 or more days. After this time they were removed, air dried, and readied for spotting.

Three spots are placed on each sheet 9 cm. from one end, with one spot being placed in the center and the other two 1.6 cm. from either edge. Slits 0.1 cm. wide are cut lengthwise along the paper beginning at the starting point of the applied spots and running to 5 cm. from the farthest end. This is done to prevent the possible merging of the materials as the chromatograms develop.

The papers are placed in a tank 30 by 54 by 21 cm. (see Fig. 1) in troughs supported by a stainless steel support held at the desired level in the tank by its own spring action. The lower layer of the solvent system (stationary phase) is then introduced into the tank on its bottom to a depth of 2 cm. The tank is sealed with a glass lid with bored holes which are sealed with aluminum foil-covered stoppers. The lower layer is allowed to equilibrate for a period of 4 to 5 hours. At the end of this period the mobile phase is introduced into the troughs containing the papers through the holes in the lid with the aid of a funnel. The holes are sealed, and the papers allowed to develop for a period of 20 hours. The temperature ranged from 35° to 37.5° C. and these are recorded at the beginning and end of each run. At the end of the 20-hour runs, the papers are removed and allowed to air dry.

Color Development for Localization and Quantitation of the Estrogens.—The color is developed by dipping the chromatograms in a solution of a mixture of equal parts of 1 per cent solutions of $FeCl_3$ and $K_4Fe(CN)_6$ in water.[8] This mixture is freshly prepared just prior to dipping. The resulting color which develops in the areas where the steroids are located will be a deep blue (Turnbull Blue). Immediately after dipping and color development, the papers are washed in dilute hydrochloric acid (10 per cent v/v) and then washed in water. While the papers are still wet, the spots are outlined with a sharply pointed soft lead pencil. The papers are then allowed to dry. Though it has been reported by Dao[3] and Meyer[8] that the spots are permanent, there is a tendency for bleaching to occur at the periphery with drying and the edges are not as clearly defined as when wet; thus, it is necessary to outline the spots while the papers are still wet. It has been found that blotting with a towel hastens drying and does not affect the color or the outlines on the chromatograms.

Quantitation of the Estrogens.—For quantitation, the outlines are preferably made by the same individual in an attempt to eliminate personal variables. After drying the papers, the area of the spots is measured with a polar planimeter. The circumference of each spot is measured twice and the average recorded. The readings are then translated into area in centimeters squared using the conversion constant predetermined for the planimeter in use.

To check the amount of steroids lost during extraction and separation, recovery experiments were carried out as follows: pooled 48-hour urine specimens were divided in half. To one half, known amounts of pure estrogens were added in quantities varying from 10 to 30 μg.

*Obtained from The Sigma Chemical Company, St. Louis, Mo.

Each half was hydrolyzed, extracted, and treated identically. The washing was done in the same manner as described by Dao[3] with a single modification—that being washing the final ether extract with activated charcoal (Darco), filtering, and washing the filter paper and charcoal several times with anhydrous ether. The extracts were taken to dryness and taken up with a known volume of redistilled methanol. Chromatograms of the same urine extract were also run with and without the addition of known amounts of the various estrogens to the spot of urine extract. The chromatograms of a given urine extract with and without the addition of the pure estrogens were run on the same paper. The data obtained from these experiments show the recovery rates to be 97, 94, and 45 to 67 per cent for estrone, 17-β-estradiol, and estriol, respectively. In these experiments, 16-epiestriol sometimes interfered with the quantitation of estriol. It was found, however, that it is possible to separate these two steroids adequately if the equilibration period is extended to 10 hours. This increases the Rf values of the estrogens; therefore, it is possible that the estrone may be eluted from a paper of the dimensions we describe.

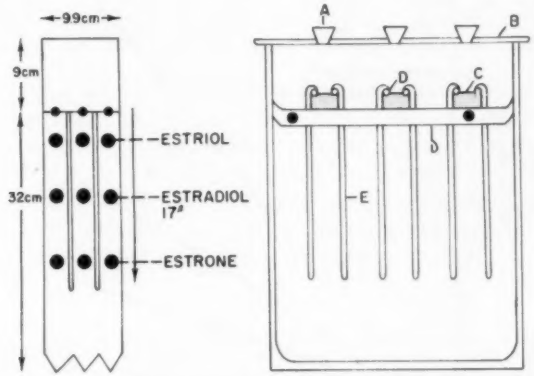

Fig. 1.—Line drawing of chromatography developing tank and chromogram. A = aluminum foil-covered stopper. B = glass lid. C = trough consisting of stainless cradle, glass boat, and 2 antisiphon rods. D = stainless steel support. E = chromatogram.

A second method which could be used to separate 16-epiestriol and estriol is that of employing a second solvent system. The substances can be eluted from the original chromatogram before color development with absolute methanol, which is evaporated to dryness. The residue is redissolved in a known quantity of methanol and rechromatographed using benzene-methanol-water (2:1:1) with the organic phase of the mixture used as the mobile phase.[9]

Although we are aware of the importance of quantitating 16-epiestriol, at the time of this writing the pure form of this steroid was unavailable to us. Since then, we have been able to acquire a sufficient quantity for a quantitative determination using the method described herein. This analysis is presently being undertaken.

Several experiments were carried out using beta glucuronidase (yeast) for enzymatic hydrolysis of urine specimens. Although the number of recoveries done using this method were not sufficient to make a critical evaluation, the chromatograms resulting from these urines showed no streaking and good separation. Since acid hydrolysis also releases unwanted chromogens, selective hydrolysis as accomplished with enzymes would minimize this undesirable characteristic of urine extracts. Extracts prepared in this way are acceptable for quantitation using the method described herein. Since, however, we are interested in a method for routine clinical application where more rapid determinations are desirable, we have found that acid hydrolysis combined with absorption of chromogens by activated charcoal during the washing procedure is as efficacious as enzymatic hydrolysis in producing clean extracts for chromatographing.

RESULTS AND CONCLUSIONS

Although there has been accumulated a vast amount of evidence supporting the premise that the area measurements can be used in quantitating small amounts of material by paper chromatography[6, 7] the method has been applied relatively infrequently for steroid determinations.

We have applied this method to separation and quantitative assay of purified naturally occurring estrogens (estrone, 17-β-estradiol, and estriol) and have obtained the results outlined below.

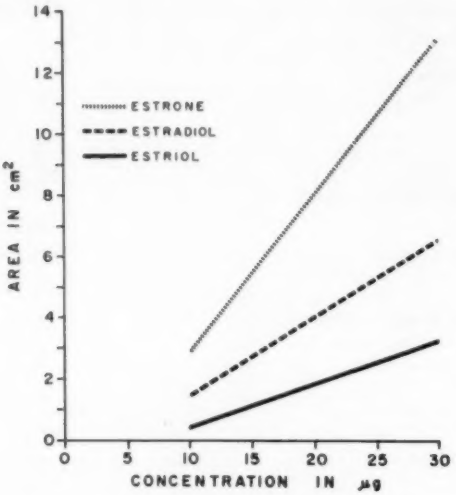

Fig. 2.—Relationship between concentration of estrogens and area of the chromatogram spot.

Our data show that the area can be used to measure the concentration. Although the spots were not always the same size on different papers for a given amount of standard (due to nonuniformity of the paper and minimal temperature variations) when equal amounts of standard were run in triplicate on the same paper, the areas of the spots were invariably the same as measured by the planimeter.

TABLE I. RELATIONSHIP BETWEEN CONCENTRATION AND AREA OF THE RESULTING SPOT

	10 µg	15 µg	20 µg	25 µg	30 µg	Rf
Estrone						0.54
Average area in cm.²	3.40	4.51	8.28	11.21	12.44	
Average standard deviation	0.28	0.21	0.52	0.82	0.30	
17-β-Estradiol						0.18
Average area in cm.²	1.30	2.57	4.41	5.12	6.27	
Average standard deviation	0.24	0.31	0.36	0.47	0.64	
Estriol						0.12
Average area in cm.²	0.87	1.25	1.80	2.42	2.79	
Average standard deviation	0.20	0.28	0.33	0.28	0.26	

Table I shows average areas and average standard deviations for the different standards in varying concentrations, and the average Rf values for each of the 3 compounds. These data compare favorably with that of Bloch and associates.[2] For estrone, however, we found our greatest deviation at 25 μg in comparison to 20 μg.

Next, we attempted to show that the area of the spot increased in proportion to the concentration. Using the least-square method, we obtained the results seen in Table II and Fig. 2.

TABLE II. AREAS AT VARYING CONCENTRATIONS AS CALCULATED BY THE LEAST-SQUARE METHOD AND COEFFICIENTS OF VARIATION (CV)

	10 μg	15 μg	20 μg	25 μg	30 μg	CV
Estrone	2.84	5.40	7.97	10.53	13.09	9.00
17-β-Estradiol	1.39	2.66	3.93	5.20	6.48	10.00
Estriol	0.43	1.11	1.83	2.52	3.22	3.90

It is apparent from these calculations that our data do not indicate that the area is proportional to the logarithm of the concentration, but is proportional to the concentration itself when using the method described. With the standard deviations shown in Table I and the coefficients of variation of 10 or less as seen in Table II, we feel that our technique is a valid method for the separation and quantitation of estrone, 17-β-estradiol, and estriol. The steroids analyzed were obviously in an unconjugated form.

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AN AUTOMATIC FLOW SWITCH

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A SIMPLE, inexpensive, time actuated switch for changing fluid flow has been devised and used in this laboratory. It is capable of stopping flow from one reservoir and starting that from another at any desired time and has been used in connection with gradient elution ion exchange chromatography such as that described by Hurlbert and his colleagues.[1]

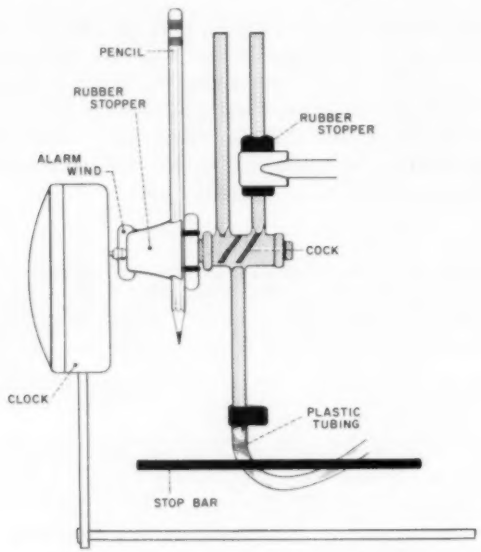

Fig. 1.—A diagrammatic side view of the apparatus.

Procedure.—A pencil was carved to a shape which fit firmly against the curved surface of the cock handle of a three-way stop cock (Corning No. 7380), and the wood and glass were joined with several wrappings of cellophane tape. A rubber stopper (size 7) was grooved across its larger end, the groove passing through the center of the flat surface; it was sufficiently large to allow the pencil to lie snugly within it. Centered on the flat surface of

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the smaller end of the stopper, a hole was cut of a shape and size appropriate to admit the alarm winding key of an ordinary mechanical alarm clock with a fairly strong alarm spring.

The stop cock was supported in a rubber shod clamp and the clock was positioned with an adjustable clamp.

In operation the alarm is wound and the key is allowed to turn until it is in a position to be fitted into the hole in the rubber stopper. The alarm is then set for the desired time and a bar is clamped into a position such that the pencil will strike it after the cock has been turned by the alarm key through the desired arc. The axes of the alarm key, the rubber stopper, and the stop cock should be carefully aligned.

The rubber clamping and stopper take up the twisting force of the alarm key satisfactorily, and there is no further movement until the device is reset.

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DETERMINATION OF GLYCEROL IN BLOOD-GLYCEROL MIXTURES

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THE addition of glycerol to blood prevents the destruction of erythrocytes caused by freezing and makes possible the preservation of whole blood at temperature below freezing for long periods of time.[1, 2] Studies in this laboratory on the mechanism whereby glycerol exerts its protective action have required the quantitative determination of glycerol in blood-glycerol mixtures. It has also been shown that glycerol solutions can be safely injected intravenously into animals and man.[3] Investigations of these infusions have also required the determination of glycerol in blood. Blood, whether freshly drawn or preserved, contains a significant amount of glucose; therefore, the analytic problem presented was to determine glycerol in the presence of glucose.

Available methods for the determination of glycerol are of 3 types: (a) the acetin method based on the esterification of glycerol by acetic anhydride; (b) colorimetric methods based on the partial oxidation of glycerol and subsequent reaction with a leuco dye; (c) oxidative methods using dichromate, cerate, or periodate. The acetin method is not suited to samples of low glycerol content and the colorimetric methods are in general neither specific nor precise. Of the oxidative agents, periodate is most convenient and specific since it acts only on compounds which contain hydroxyl groups on neighboring carbon atoms.[4] However, with this agent, as with all others, glucose is an interfering substance. The present study was directed toward developing a method of determining glycerol by periodate oxidation in which the interference by glucose could be eliminated or for which an adequately precise correction could be made.

Glucose can be removed by precipitation with barium or calcium hydroxide and alcohol.[5] This procedure requires overnight refrigeration and is tedious and not very reliable. Glycerol has been determined in the presence of glucose in fermentation mixtures by cerate oxidation of both glycerol and glucose followed by an independent determination of glucose.[6] This method is not applicable to cases where the glycerol content is considerably higher than the glucose content as is the case in the blood-glycerol mixtures the analysis of which is desired. Furthermore, it involves two different types of nonstoichiometric determinations which are not of comparable precision. The determination of glycerol in blood has been performed by a method which depends on the fact that glycerol is more rapidly oxidized by periodate than is glucose.[7] This

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method requires precise control of conditions of oxidation and the subsequent colorimetric determination of the resulting formalin is troublesome because iodide must be removed, light must be excluded, and freezing mixtures must be used to control temperature during addition of concentrated sulfuric acid. Glycerol has been separated from glucose by column chromatography[8, 9] but such a technique is hardly suited to rapid routine analysis.

The present method is based on the fact that when heated with aqueous alkali the glucose molecule is disrupted and most of its reducing action is lost, but the glycerol molecule is unaffected. Keppel[10] had reported a procedure for determining glycerol in egg yolk preparations which contained very small amounts of glucose by boiling the sample with alkali prior to the determination of glycerol by periodate oxidation. This treatment with alkali eliminated about 65 per cent of the reducing action of the glucose and the remaining interference was neglected because the error was small. However, the author stated that if more than minute quantities were present, the glucose must be removed prior to treatment with periodate. The present investigation began with an attempt to eliminate the residual reducing action which is present after treating glucose with alkali. Attempts to achieve this by varying the experimental conditions, by extraction with organic solvents, or by selective oxidation with agents other than periodate were not successful. However, it was found that the fraction of the reducing action of glucose which was destroyed by treatment with alkali was large enough and constant enough so that an adequately precise correction could be made for the residual reducing activity. On this basis, a method has been elaborated in which identical aliquots of a solution containing both glycerol and glucose are oxidized by an excess of periodate both before and after treatment with alkali. From the two titrations of the excess periodate a moderately precise value of the glycerol content is obtained and a somewhat less precise value of the glucose content is also obtained.

MATERIALS AND METHODS

Solutions and Reagents.—

Periodate 0.02 M: Prepare 0.1 M sodium periodate by dissolving 21.4 Gm. of $NaIO_4$ in 1,000 ml. of water. As needed, 0.02 M. periodate is made by diluting the 0.1 M solution with water.

Standard arsenite: Prepare 0.1000 N arsenite by dissolving 4.9465 Gm. of analytic reagent grade As_2O_3 in 40 ml. of normal sodium hydroxide solution, adding normal hydrochloric acid until the solution is faintly acid to litmus and diluting to 1,000 ml. in a volumetric flask. The 0.02 N solution is made by diluting the 0.1 N solution with water.

Glycerol and glucose solutions: These solutions were prepared from weighted quantities of the analytic reagent grade material which were diluted to give a convenient concentration. They were assayed by the periodate oxidation method described herein.

Preparation of Protein-free Filtrate.—Transfer an accurately weighed sample of blood (containing a total of not more than 0.4 Gm. of glycerol and glucose) to a 50 ml. beaker and add 5 ml. of water to lyse the cells. Precipitate the protein by adding dropwise with mixing 6 ml. of cold 20 per cent trichloroacetic acid solution and allow to stand for one hour at 5° C. Filter through a paper (which has been wet with 10 per cent trichloroacetic

acid) into a 100 ml. volumetric flask. Wash the beaker, precipitate, and paper 3 times with 5 ml. portions of 10 per cent trichloroacetic acid. Dilute the filtrate to the mark with water, mix, and take aliquots for the determinations described below.

Titration of Untreated Aliquot.—Transfer a 10 ml. aliquot of the protein-free filtrate to a 100 ml. volumetric flask, add one drop of bromeresol purple indicator (0.1 per cent in alcohol) and then 10 per cent NaOH until just alkaline. Add from a pipette 50 ml. of 0.02 M sodium periodate solution, mix, and allow to stand for two hours. Similarly prepare a "blank" determination by using 10 ml. of water in place of the aliquot. After adding the periodate the solutions should be acid to the indicator; if not, acidify with dilute sulfuric acid. After standing 2 hours, dilute to the mark and mix. Transfer 20 ml. aliquots to 125 ml. Erlenmeyer flasks, add 10 per cent $Na_2HPO_4 \cdot 12H_2O$ solution until *definitely* alkaline (about 10 ml.) and then 0.5 Gm. of KI crystals. Titrate the iodine which is formed with standard 0.02 N arsenite solution using starch solution as indicator. The titration must be conducted slowly near the end point.

Titration of Alkali-Treated Aliquot.—Transfer a 10 ml. aliquot of the protein-free filtrate to a 125 ml. Erlenmeyer flask, add one drop of bromeresol purple indicator and then 10 per cent NaOH until neutrality and then 2 ml. in excess. Heat to a gentle boil and boil for one minute. Do not mistake the release of CO_2 from the decarboxylation of trichloroacetate as boiling. Allow to cool for 5 minutes, add 1:1 H_2SO_4 until acid and then 2 drops in excess. Boil gently for one minute, then cool the flask in cold water. Add 10 per cent NaOH until just alkaline and transfer the solution quantitatively to a 100 ml. volumetric flask. Add periodate and complete the determination as for the untreated aliquot.

Calculation.—

("Blank" titration)—(untreated aliquot titration) = A ml.
("Blank" titration)—(alkali-treated aliquot titration) = B ml.

It has been found that after alkali treatment 18.5 per cent of the original reducing activity of the glucose remains and the glycerol is not affected.

Let G = ml. of arsenite equivalent to glycerol; D = ml. of arsenite equivalent to glucose (dextrose). Then

$$A = G + D \quad (1)$$
$$B = G + 0.185\ D \quad (2)$$
$$A - B = 0.815\ D$$
$$D = 1.227\ (A - B)$$

Substituting this value of D in equation 1 gives: G = 1.227 B − 0.227 A. 1.00 ml. of 0.02 N arsenite is equivalent to 0.4605 mg. of glycerol. G × 0.4605 = mg. of glycerol in aliquot, or G × 4.605 = mg. of glycerol in original sample. *Milligrams of glycerol in original sample* = 5.650 B − 1.045 A.

The quantity of glucose present can also be calculated: D = 1.227 (A − B). 1.00 ml. of 0.02 N arsenite is equivalent to 0.3603 mg. of glucose. D × 0.3603 = mg. of glucose in aliquot. D × 3.603 = mg. of glucose in original sample. *Milligrams of glucose in original sample* = 4.420 (A − B).

RESULTS AND DISCUSSION

It was found that maximal removal of the reducing action of glucose was obtained by boiling the alkaline solution for 30 seconds and that longer boiling had no further effect. A boiling period of one minute was adopted to provide a safety factor.

A number of independent experiments were performed to determine what percentage of the reducing action of glucose was destroyed by treatment with alkali as described in the procedure above. Five or more such experiments were

done for each of 3 different quantities of glucose per reaction vessel. With 1.34 mg. of glucose per flask, the percentage of the reducing action destroyed was 81.1 with a standard deviation of 1.1; with 2.68 mg. of glucose these values were 81.8 and 0.9, respectively; with 4.02 mg. of glucose they were 81.3 and 1.4. It was apparent that the percentage destroyed, within the experimental error, is independent of the amount of glucose present. The average for all the experiments was 81.5 with a standard deviation of 1.0, and it is this value which has been used in the preceding method of calculation. It was also found that the quantity of glycerol present did not influence the destruction of glucose by heating with alkali.

TABLE I. DETERMINATION OF GLYCEROL AND GLUCOSE IN SIMPLE SOLUTIONS

AMOUNT PRESENT (MG.)		AMOUNT FOUND (MG.)		PER CENT ERROR	
GLYCEROL	GLUCOSE	GLYCEROL	GLUCOSE	GLYCEROL	GLUCOSE
6.43	6.70	6.30	7.25	-2.0	+8.2
6.43	13.40	6.40	13.30	-0.5	-0.7
6.43	20.10	6.60	19.60	+2.6	-2.5
12.86	6.70	12.86	6.45	0.0	-3.7
12.86	13.40	12.70	13.30	-1.2	-0.7
12.86	20.10	13.35	19.50	+3.8	-3.0
19.29	6.70	19.35	6.40	+0.3	-4.5
19.29	13.40	19.40	13.00	+0.6	-3.0
19.29	20.10	19.17	19.43	-0.6	-3.3
25.72	6.70	25.75	6.25	+0.1	-6.7
25.72	13.40	25.10	13.55	-2.4	+1.1
25.72	20.10	25.85	18.70	+0.5	-7.0
32.15	6.70	32.25	6.05	+0.3	-9.7
32.15	13.40	31.60	13.35	-1.7	-0.4
32.15	20.10	32.60	19.75	+1.4	-1.7

The method was tested at first with simple solutions containing known quantities of both glycerol and glucose. Since no protein was present the treatment with trichloroacetic acid was omitted. The results of these analyses are shown in Table I. The ratio of glycerol to glucose in these mixtures varied from about 0.3 to 5.0. Although the error in the determination of glycerol is not a simple function of this ratio, it is evident that the error is smaller when this ratio is large. The standard deviation in this group for the determination of glycerol was 1.3 per cent of the amount present. The standard deviation in determining small quantities of glycerol by the periodate method in solutions containing only glycerol is of the order of ±1 per cent of the amount present. Thus, the present method for determining glycerol in the presence of glucose compares favorably with the determination of glycerol in pure solution. As is to be expected, the results for glucose content were less precise, the standard deviation being ±4.8 per cent of the amount present.

The complete procedure was tested with samples prepared by adding known quantities of glycerol and glucose to human blood which had been collected into acid citrate dextrose (ACD) preservative-anticoagulant solution. Individual samples for analysis were prepared by adding carefully measured volumes of glycerol and glucose solutions to 4.0 ml. portions of ACD blood.

Since ACD blood contains glucose, the amount of glucose present was the sum of glucose in the blood and the glucose added. The results of the analyses of these blood samples are shown in Table II. These results also show that the error in the determination of glycerol is in general lower when the ratio of glycerol to glucose is higher. The standard deviation for all the analyses shown is ±2.3 per cent of the amount present. The spread in the results for glucose is larger, but if the samples containing the lowest quantity of glucose (those to which no glucose was added) are excluded from the calculation, the standard deviation is ±4.6 per cent of the amount present.

The precision of the results obtained with the present method compares quite favorably with other methods for the determination of glycerol in the presence of interfering substances. It has the advantages of considerable simplicity since it requires no separations and uses only a single type of titration. It requires no special glassware or expensive reagents. It further provides a value for the glucose content of the sample with no additional labor.

The present method should be directly applicable to the determination of glycerol in the presence of fructose and mannose, the epimers of glucose whose behavior in alkaline solutions should be identical with that of glucose. The method should also be applicable in the presence of other reducing sugars, but the optimal conditions and the reproducibility would have to be established. The method could also be used for the determination of glycols or other compounds with vicinal hydroxyl groups in the presence of reducing sugars.

TABLE II. DETERMINATION OF GLYCEROL AND GLUCOSE IN BLOOD SAMPLES

AMOUNT PRESENT (MG.)		AMOUNT FOUND (MG.)		PER CENT ERROR	
GLYCEROL	GLUCOSE	GLYCEROL	GLUCOSE	GLYCEROL	GLUCOSE
25.1	12.7	24.2	13.8	−3.6	+8.7
25.1	37.6	23.9	39.4	−4.8	+4.8
25.1	37.6	24.7	37.9	−1.6	+0.8
50.2	12.7	49.8	14.1	−0.8	+11.0
50.2	12.7	50.2	13.0	0.0	+2.4
50.2	12.7	50.4	12.6	+0.4	−0.8
50.2	37.6	50.9	34.4	+1.4	−8.5
50.2	37.6	48.6	37.5	−3.2	−0.3
50.2	37.6	49.4	39.7	−1.6	−5.6
50.2	62.6	50.0	61.6	−0.4	−1.6
100.4	12.7	100.7	10.5	+0.3	−17.3
100.4	37.6	99.0	35.5	−1.4	−5.6
100.4	62.6	96.6	63.0	−3.8	+6.4
100.4	62.6	99.5	60.0	−0.9	+4.2
200.8	62.6	200.0	60.6	−0.4	+4.2
200.8	112.4	196.0	112.0	−2.4	−0.4

SUMMARY

A relatively simple method has been elaborated for the determination of glycerol in blood-glycerol mixtures which contain glucose. The method is based on the fact that the glucose molecule is disrupted on heating in alkaline solution and a large and reproducible fraction of its reducing activity is lost. The method is carried out by determining the amount of periodate reduced by aliquots of a protein-free filtrate containing glycerol and glucose both before

and after treatment with alkali. From the two titrations the glycerol content can be calculated with a mean error of ±1.4 per cent. From the same titrations a somewhat less precise value of the glucose content can be calculated. The method has general applicability to solutions containing glycerol and glucose and should be applicable to mixtures of related compounds such as glycols and other reducing sugars.

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A METHOD FOR RECOVERING LARGE NUMBERS OF PLATELETS AND LEUKOCYTES FROM NORMAL HUMAN BLOOD

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WE WISH to report a method for recovering large numbers of platelets and leukocytes from normal human donors. We believe that the method is particularly applicable where fresh plasma is prepared on a large scale.

MATERIALS AND METHODS

Our source of platelets and leukocytes from normal human donors has been the cellular mass remaining after the separation of plasma for use as antihemophilic plasma. The plasma was drawn off within 3 hours of the withdrawal of blood from donors.

Five hundred milliliter quantities of blood were drawn from normal human donors into vacuum bottles containing 50 ml. of 4 per cent sodium citrate. This blood was centrifuged at approximately 1,500 g (middle of bottle) for 45 minutes at 4° C. The buffy coat was a 1 to 2 mm. layer between plasma and erythrocytes. The plasma was removed by aspiration and processed further for antihemophilic activity. In 15 per cent of the units of bloods, the cells in the buffy coats were clumped markedly; these were not used.

The method for recovering the leukocytes from this centrifuged blood depended upon a device for aspirating the buffy coat. This device was a spoon on a long handle, along which ran a plastic tube. The spoon was made by riveting a curved handle to an ordinary aluminum measuring teaspoon, from which a section had been removed (Fig. 1).

In operation, a vacuum led through a trapping flask to a collecting tube, which in turn was connected to the aspirating spoon (Fig. 1). The collecting tube was a Pyrex culture tube, 20 by 150 mm. A glass tube (3.5 mm. diameter) led to the plastic tubing (3.5 mm. inside diameter) of the spoon assembly.

The buffy coat (platelets and leukocytes) was then aspirated (Fig. 1). The spoon lip was placed just beneath the buffy layer to avoid excessive erythrocyte contamination. The vacuum was controlled by pinching the tubing in order to assure a slow, steady flow rate without foaming.

The buffy coat so obtained was a mixture of plasma and formed elements in a volume of 20 to 40 ml. Further separation was accomplished by recentrifugation of the collecting tube at 200 g (middle of tube) for 30 minutes at 4° C. This centrifugation yielded 4 layers: plasma, platelet, buffy (mainly leukocytes), and erythrocyte (Fig. 2). (The "buffy layer" from this second centrifugation should be distinguished from the original "buffy coat.") These layers were individually aspirated with a syringe and needle (3 in., 18 gauge).

Aliquots were examined microscopically. All steps in the procedure were performed aseptically. Nonsiliconized glassware was used throughout, since siliconization did not improve the yield. One person was regularly able to recover the leukocytes from 10 to 25 pints of blood within a period of 3 hours.

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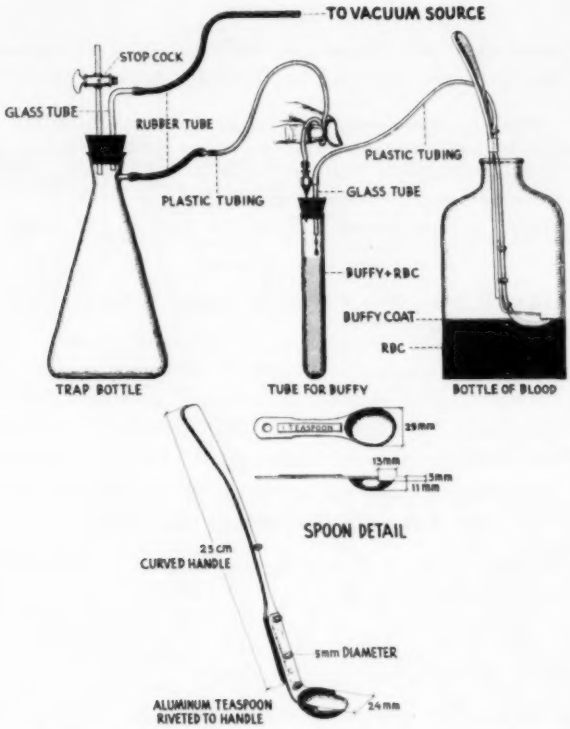

Fig. 1.—Suction spoon method for removal of buffy coat and platelets.

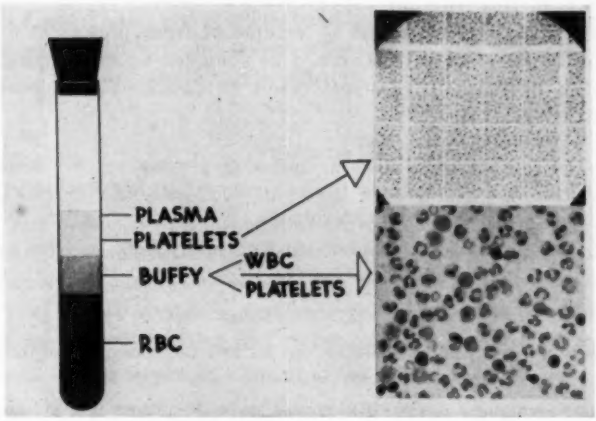

Fig. 2.—Recentrifuged buffy coat showing platelet layer, buffy layer, and red blood cells.

RESULTS

The compositions of the platelet and buffy layers of 19 representative, recentrifuged specimens are recorded in Table I. Approximately 60 per cent

TABLE I. COMPOSITION OF PLATELET AND BUFFY LAYERS

		PLATELET LAYER		BUFFY LAYER	
		AVERAGE	RANGE	AVERAGE	RANGE
		7*	2.5-13	3.5	2.2-5
Packed cell volume	Platelets and leukocytes	7.5%	4-14%	51%	42-58%
	Erythrocytes	†	†	1.0%	0.5-2.0%
Formed elements (thousands per mm.^{3})	Leukocytes	1.2	0.2-3.1	600	450-990
	Platelets	6,600	3,000-16,000	11,500	9,000-17,000
	Erythrocytes	†	†	185	40-450
Absolute numbers of formed elements (billions)	Leukocytes	0.009	0.001-0.028	2.2	1.2-3.8
	Platelets	53	20-109	43	19-60
	Erythrocytes	†	†	0.65	0.2-1.8

*Total volume, milliliters.
†Not enough to measure.

of the leukocytes in the original 500 ml. of blood was recovered in an average volume of 3.5 ml. The following observations were made on these layers.

A. Platelet Layer.—The platelets in this layer were well separated; however, those obtained in the buffy layer were occasionally clumped. The platelets were found to function normally both in vitro,* by recalcification time, plasma clot retraction, and thromboplastin generation, and in vivo, by platelet count and clot retraction, in thrombocytopenic patients.

B. Buffy Layer.—The leukocytes in this layer were not clumped microscopically. They were motile and appeared morphologically intact by direct and phase microscopy. As noted in Table I, there were some erythrocytes and many platelets in this fraction. Most of the remaining erythrocytes could be removed by recentrifugation from ½ volume of fresh plasma in a centrifuge tube. A further decrease in RBC could be achieved by recentrifugation with plasma, and a decrease in platelets could be obtained by recentrifugation with EDTA.

SUMMARY

1. A method is described for the recovery of leukocytes and platelets in large quantities from normal human donors.

2. The platelets so isolated functioned normally both in vitro and in vivo.

3. The leukocytes so isolated were motile and intact as judged by direct and phase microscopy.

We wish to express our gratitude to The Hyland Laboratories, Los Angeles, Calif., for the generous use of their facilities, and to Dr. John W. Palmer for his cooperation.

*These tests were kindly performed by Doctor Sherwood Miller.

LIQUID SCINTILLATION COUNTING OF HISTAMINE-C^{14} AND ITS METABOLITES

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WITH THE TECHNICAL ASSISTANCE OF SUE R. STEVENS

AROMATIC solutions of certain phosphorescent substances emit light in the presence of beta radiation. Detection of this light emission with a photomultiplier tube provides an efficient and sensitive method for the assay of the low-energy beta radiation emitted by such isotopes as C^{14}, S^{35}, and H^3.

The advantages of such liquid scintillation techniques have been: high efficiency, ability to assay relatively large volumes of sample, and simplicity of sample preparation. The most important problems to be overcome have involved the high background counting rates dues to thermionic emission from the photomultiplier tube, and the insolubility of aqueous samples in aromatic hydrocarbons, such as toluene, which are the most efficient scintillating solvents. Innovations in instrumentation, including refrigeration of the photomultiplier tubes, have reduced background counting rates to satisfactory levels. However, techniques for incorporation of aqueous samples into organic scintillation solutions have not been applicable to new situations without modification.

When beta radiation assay of a labeled substance in biologic fluids is desired, one may prepare a derivative soluble in toluene. Thus, certain materials such as CO_2, amino acids, and even proteins have been successfully incorporated into toluene with a quaternary ammonium base (Hyamine).[1, 2] The probability that urine or plasma samples would require conversion to CO_2 in order to assay total radioactivity by this method persuaded us to investigate alternative methods of preparation.

Suspension of the sample material in a thixotropic gel containing the scintillation solvent and phosphors has been used to assay finely ground solid particles.[3] The possibility of suspending an immiscible liquid in droplet form in such a gel for scintillation assay has not been adequately investigated.

Aqueous samples have been successfully dissolved in teritary liquid mixtures of methanol or ethanol and toluene,[4] but the only acceptable scintillation solvent fully miscible with water is p-dioxane.[5, 6] Purification of commercially

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available dioxane is required and the freezing point of dioxane (10° C.) is above the temperature which is optimal for liquid scintillation assay. Davidson[5] has overcome the latter difficulty by adding two other ethers to dioxane. The additional ethers, anisole and 1,2-dimethoxyethane, serve to depress the freezing point while maintaining counting efficiency.

Our objective was to develop a system requiring minimal preparation of plasma and urine samples obtained after administration of C^{14}-labeled histamine to human beings. A high degree of sensitivity was required in order to allow the administration of amounts of histamine small enough to approach physiologic conditions. The purpose of this report is to present our findings regarding the use of the above-mentioned methods in satisfying these requirements.

MATERIALS AND METHODS

*Histamine-C^{14} Dihydrochloride.**—This material was dissolved in 0.9 per cent NaCl and 0.2 per cent glucose to a concentration of 0.85 mg. per milliliter† and stored at −20° C. Chemical analysis of this stock solution several times in the course of a year showed no change in histamine concentration. Periodically, aliquots were diluted with 0.9 per cent NaCl to appropriate concentrations, and passed through Seitz filters into sterile vials.

Since histamine is strongly adsorbed by cellulose, the Seitz filtration removed a certain amount of histamine from the saline solutions. Seitz filtration of solutions of histamine in water showed far greater adsorptive losses. It was obviously necessary to repeat the assay of all solutions after filtration.

Scintillation Assay.—Samples were placed in screw-top glass vials‡ and counted with a Packard Tri-Carb liquid scintillation spectrometer.§ Handling of the glass vials was kept to a minimum, and they were wiped carefully before counting. Photoactivation of the glass vials producing spurious counts was occasionally detected[5]; this necessitated storage of such samples in the dark for 6 to 12 hours before counting.

The temperature of the sample chamber, photomultiplier tubes, and preamplifiers was maintained at 0° C. The high voltage applied to the photomultiplier tubes was selected to provide the highest ratio of the square of the sample count to the background count for the particular solvent system in use. Discriminator controls were set to count a "window" of pulse energies from 10 to 70 volts, thereby decreasing background counts and minimizing the effects of minor shifts of the pulse energy spectrum.

A background count was obtained over a 30-minute period twice daily. Samples containing more than 70 disintegrations per minute (d.p.m.) were counted long enough to provide an accuracy of ± 5 per cent at the 0.9 confidence limit. Samples with less than 70 d.p.m. were counted for one hour. The statistical limits were obtained from the nomograms of Jarrett.[8]

*Obtained from Nuclear Chicago Corporation, Chicago 10, Ill., labeled in the 2 position of the imidazole ring. The manufacturer's assay was 4.75 mc. per millimole of histamine dihydrochloride. Assay in our laboratory by chemical determination of histamine content,[7] combined with gas-flow Geiger counting (following conversion to $BaCO_3$), revealed a specific activity of 4.52 mc. per millimole of histamine dihydrochloride. Purity was confirmed by paper chromatography.

†All references to histamine concentration are in terms of the base unless otherwise stated.

‡Wheaton Glass Co., Millville, N. J. These vials were made with low-potassium glass and the plastic caps were lined with polyethylene.

§Packard Instrument Company, La Grange, Ill. Photomultiplier "noise" due to thermionic emission was reduced to a minimum by cooling, by utilizing two photomultiplier tubes and a coincidence circuit (so that photons must strike both photomultiplier tube faces at nearly the same time to be counted), and by the use of discriminator circuits which could be set to reject those pulse energies most likely due to thermionic emission.

Preparation of Protein-Free Plasma Extracts.—Venous blood was obtained in tubes containing a balanced oxalate anticoagulant. The red cells were separated by centrifugation and the plasma deproteinized by the addition of 0.20 volume of 30 per cent trichloroacetic acid (TCA). The supernatant solution, which contained all the radioactivity, was then buffered by the addition of 0.10 volume of 4 M sodium acetate.

Gel Suspension Counting.—It was hoped that water droplets might be suspended in a gel, thus circumventing the problems of sample solubility. Twenty-five grams of glyceryl tri-12-hydroxystearin (Thixcin*) was dissolved in 100 ml. toluene containing 0.7 Gm. diphenyloxazole (PPO†) and 0.05 Gm. 1,4-di-(5-phenyloxazolyl)benzene (POPOP†) as scintillation phosphors. These ingredients were mixed in a Waring Blendor at low speed for one hour. Water droplets in the gel remained liquid at 0° C., but were frozen at −15° C. Samples were prepared with water, urine, and protein-free plasma extracts. Suspensions of sample varied from 0.1 to 2 ml. in 20 ml. of the gel. A scintillation counter setting of 1,000 volts was used for the photomultiplier tubes in assaying these suspensions.

Polyether 611.—This mixture of ethers was developed by Davidson as a solvent for assaying labeled purines and pyrimidines.[5] The solvent contained 6 parts p-dioxane, 1 part anisole, and 1 part 1,2-dimethoxyethane. To this were added, as scintillation solutes, 1.2 Gm. per 100 ml. PPO and 0.04 Gm. per 100 ml. POPOP. It has been suggested that all 3 solvents be specially purified in order to improve the counting efficiency.[5] We found that the dioxane needed purification, but reagent grades of anisole and 1,2-dimethoxyethane were adequate without further preparation.‡ Purification[9, 10] of dioxane increased the counting efficiency of standard histamine-C14 solutions from 30 to 57 per cent.

Eight milliliters of polyether 611 dissolved 2 ml. of water or ethylene glycol, but would not dissolve an equal amount of urine, protein-free plasma extract, or physiologic saline. Accordingly, each plasma extract was diluted fourfold with water and 1 ml. of this solution was added to 8 ml. of the polyether 611 in a counting vial. Urine was diluted tenfold with water and 1 ml. of the diluted urine dissolved in 8 ml. of polyether 611. A scintillation counter setting of 1,080 volts on the photomultiplier tubes was found to be most efficient for these assays.

Internal Standards.—Due to the variations induced by quenching of phosphorescence by certain sample solutions, internal standards are commonly used in liquid scintillation counting.[11] For such use, we prepared a standard solution of histamine-C14 in 0.9 per cent NaCl which initially contained 1 μg per milliliter. One-tenth milliliter of this solution plus 0.8 ml. of polyether 611 were added to each previously counted sample, and the total sample was reassayed immediately. Just before its use as an internal standard, each standard solution was reassayed.

RESULTS

Gel Suspension.—When an aqueous sample was shaken in the thixotropic gel, a generalized dispersion of small droplets occurred. Over a period of hours the droplets gradually coalesced and settled. These droplets varied considerably in size. More vigorous or prolonged shaking usually resulted in smaller droplets and higher counting efficiency. The counting rates obtained, however, varied unpredictably with as much as 100 per cent error. Careful control of the force and duration of shaking in making the suspension as well as the length of standing before counting failed to eliminate this difficulty. The counting

*Baker Castor Oil Company, New York, N. Y. This substance dissolved in toluene forms a thixotropic gel which is stable between pH 3 and 10.

†Pilot Chemicals Corporation, Waltham, Mass.

‡All 3 reagents were obtained from Distillation Products Industries, Eastman Organic Chemicals, Rochester 3, N. Y.

efficiency of this method varied inversely with the amount of water suspended. A maximum efficiency of 50 per cent was obtained with 0.1 ml. of aqueous sample suspended in 20 ml. of gel.

Polyether 611.—

Standard solutions: When standard solutions of histamine-C^{14} in saline were placed in the counting vials with the scintillation solution, the efficiency of the counting method was 58.6 per cent.

The counting rate of more dilute solutions was less than the expected value for the amount of radioactive material present (Table I). The same phenomenon was more pronounced when similar solutions were not counted immediately (Table II). Addition of carrier histamine to the sample solution prevented the drop in counting rate. These findings were attributed not to changes in counting efficiency, but to the adsorption of histamine by glassware. As a result of this information internal standards were always reassayed just before use.

TABLE I. LIQUID SCINTILLATION ASSAY OF STANDARD SOLUTIONS OF HISTAMINE-C^{14} USING POLYETHER 611

HISTAMINE-C^{14} (μg)	CALCULATED D.P.M.	CALCULATED C.P.M.* AT 58.6 % EFFICIENCY	OBSERVED C.P.M.-BKG† ± 0.9 ERROR
0.85	75,990	44,533	44,533 ± 110
0.085	7,599	4,453	4,345 ± 35
0.0085	760	445	424 ± 11

*c.p.m. = counts per minute.

†bkg = background.

All samples contained the histamine-C^{14} in 0.1 ml. physiologic saline. This was added to 0.9 ml. water and 8.0 ml. polyether 611 and counted immediately. The calculated disintegrations per minute (d.p.m.) were obtained by assay of the stock solution through conversion to $BaCO_3$ and gas-flow Geiger counting. Note the loss of radioactivity in the dilute samples.

TABLE II. CHANGE IN SCINTILLATION ASSAY OF DILUTE HISTAMINE SOLUTIONS IN WATER

	C.P.M. COUNTED IMMEDIATELY	COMMENTS	C.P.M. 1 HOUR AFTER ADDITION
Ave. 1 + 2	4,179	100 γ nonradioactive histamine added to each	3,992
Ave. 3 + 4	4,157	1 γ nonradioactive histamine added to each	3,015

One-tenth milliliter of histamine-C^{14} solution plus 0.9 ml. water and 8 ml. polyether 611 are added to each of 4 vials. These were counted (column 1), and then 0.1 ml. containing 100 γ or 1 γ of nonradioactive histamine was added. Samples counted again after an hour (column 3). Note effect of carrier histamine in preventing drop in detectable radioactivity.

Plasma and Urine Samples.—There was no decrease in the counting efficiency of plasma or urine samples with time or dilution indicating that adsorption to glass was not a problem with these samples. Background counts of urine, plasma, or saline in the polyether 611 all fell in the same range. The mean of 126 such counts was 23.4 counts per minute (S.D. 1.6, Range 17.1 to 23.1).

At the level of synthetic histamine-C^{14} specific activity available to us (4.52 mc./mM), the sensitivity of the assay was such that C^{14} equivalent to 0.3 μg of histamine per 100 ml. of plasma could be detected with less than 5 per cent error (0.9 confidence limit) with one hour's counting. As seen in Table III, the counting efficiency of the plasma solutions was the same as the counting efficiency of a standard solution, that is, there was no "quenching" by any TCA-soluble components. Much of the plasma radioactivity represented metabolites of histamine. The sensitivity for detection of specific histamine radioactivity could be increased greatly by preliminary concentration and extraction of as much as 10 ml. of plasma with the method developed by Lowry and associates for the chemical assay of histamine,[7] combined with radioactive assay of the final extract (0.05 ml. in 2 N HCl). The amount of plasma radioactivity analyzed could thus be increased fortyfold, with a corresponding increase in the sensitivity of histamine-C^{14} detection to 0.0075 μg of base per 100 ml. plasma.

TABLE III. RADIOACTIVITY OF PLASMA EXTRACTS FOLLOWING ADMINISTRATION OF HISTAMINE-C^{14} (SUBJECT K. T.)

TIME	C.P.M.	BKG	C.P.M.-BKG ± 0.9 ERROR	INTERNAL STANDARD	% OF ADMINISTERED C^{14} PER 100 ML. PLASMA ± 0.9 ERROR
2 min.	63	25	38 ± 1.9	3,748	0.40 ± 0.02
7 min.	44	25	19 ± 1.5	3,721	0.20 ± 0.02
10 min.	39	25	14 ± 1.4	3,760	0.15 ± 0.02
30 min.	40	25	15 ± 1.4	3,759	0.16 ± 0.02
60 min.	38	25	13 ± 1.4	3,661	0.14 ± 0.02
24 hr.	28	25	3 ± 1.2	3,772	0.03 ± 0.01

All samples were counted for 1 hour. Subject was given 95 μg histamine-C^{14} intravenously and plasma collected at the indicated intervals. Assay of the solution used for the internal standard was 6,382 d.p.m. in 0.1 ml.

TABLE IV. URINARY RADIOACTIVITY FOLLOWING ADMINISTRATION OF HISTAMINE-C^{14} (SUBJECT K. T.)

SAMPLE (HR.)	VOL. (ML.)	C.P.M.	COUNTING TIME (MIN.)	C.P.M.-BKG ± 0.9 ERROR	INTERNAL STANDARD	EFFICIENCY	% OF ADMINISTERED C^{14} RECOVERED IN TOTAL URINE SAMPLE ± 0.9 ERROR
0-1	82	1,379	10	1,355 ± 19	3,492	53.2 %	24.58 ± .34
1-3	240	532	30	508 ± 7	3,523	53.6 %	26.73 ± .37
3-6	375	171	30	147 ± 4	3,410	52.0 %	12.48 ± .34
6-12	200	139	30	115 ± 3.6	2,919	44.5 %	5.45 ± .19
12-24	420	111	30	87 ± 3.2	3,659	55.8 %	7.70 ± .28
24-48	1,300	45	30	21 ± 2.1	3,275	49.9 %	6.43 ± .64
48-72	2,000	24	30	0	—	—	—
						Total	83.37 ± 2.16

Subject was given 95 μg histamine-C^{14}. Assay of the solution used for the internal standard was 6,562 d.p.m. in 0.1 ml. Background = 24 c.p.m.

Urine samples could be assayed with a maximum efficiency of approximately 55 per cent. The sensitivity was such that C^{14} equivalent to 0.008 μg of histamine base could be detected in 1 ml. of urine with less than 5 per cent error

at the 0.9 confidence limit when counted for one hour. Variations in efficiency were ordinarily slight, but sufficiently important to require internal standard verification (Table IV). These variations were due to the quenching effects of colored materials and salts in the urine samples. Several urine specimens obtained from patients with advanced liver or kidney disease reduced the counting efficiency to as little as 20 per cent. In a few highly colored or acidic samples where the counting efficiency was less than 20 per cent, accurate results could not be obtained with the internal standard technique. Further dilution of the sample was then necessary to provide a more satisfactory counting solution. The results of the assays done in normal and diseased subjects will be published elsewhere.

Other problems due to quenching occurred when concentrated methanol extracts of dried urine were prepared for chromatography and, in addition, were assayed in the liquid scintillation counter. The dark-colored material, although relatively salt-free, often produced severe quenching when added to the scintillation solution. Illustrative examples are shown in Table V.

TABLE V. EFFICIENCY OF LIQUID SCINTILLATION ASSAY FOR SELECTED SAMPLES OBTAINED FROM METHANOL EXTRACTS OF DRIED URINE

SAMPLE	VOL. OF EXTRACT (ML.)	C.P.M.	C.P.M. -BKG	INTERNAL STANDARD (C.P.M.)	ASSAY OF STANDARD D.P.M./0.1 ML.	EFFICIENCY	D.P.M. IN THE EXTRACT
KT #1	4.1	3,070	3,048	3,773	7,427	50.8 %	2.48×10^5
RS #1	6.0	5,738	5,716	1,966	7,175	27.4 %	1.25×10^6

One-tenth milliliter of methanol extract was added to 8.0 ml. of polyether 611 with 0.9 ml. water. Background = 22 c.p.m.

DISCUSSION

The droplet suspension of aqueous samples in a thixotropic gel proved to be an unreliable method for C^{14} assay. This unreliability was due to an uncontrollable variation in the self-absorption of radiation, counting geometry, and light scattering of the sample. An additional disadvantage this method shared with all methods of nonhomogenous counting was the difficulty in utilizing an internal standard.

The use of polyether 611 provided a successful method for assay of the urine and plasma samples, although the volume of sample which could be counted was relatively small. This limitation in volume appeared to be due primarily to the presence of inorganic salts in the sampled fluids, so that dilution with water was required. Tertiary liquid mixtures of urine, protein-free plasma, or 0.9 per cent NaCl with ethanol or methanol and toluene were entirely unsuccessful for the same reason. Thus, one advantage of liquid scintillation techniques, namely, utilization of fairly large volumes of sample, is insignificant for assaying aqueous biologic solutions.

Despite the problems of solubility, the sensitivity of the method was still sufficient to permit detection of radioactivity equivalent to 0.3 μg histamine base

per 100 ml. plasma and 8 μg per liter of urine, without requiring special procedures for sample preparation.

Extraction and concentration procedures, which would remove other components of plasma or urine from the substances of interest, should increase sensitivity as well as specificity. For example, prior concentration of plasma extracts on cation exchange columns provided a method by which histamine-C^{14} could be detected at the extremely low concentration of 0.0075 μg per 100 ml. plasma.

The use of internal standards was required for assaying urine samples because of varying degrees of quenching (Tables IV and V). Preparation of such internal standards required careful attention to solubility characteristics and adsorption of histamine-C^{14} by Seitz filters and glassware. It was necessary to reassay the solutions prior to use. Additions of carrier histamine greatly lessened this problem.

Although these studies involved the measurement of labeled histamine and histamine metabolites only, we believe the techniques should be generally applicable for low-energy beta assay of labeled compounds in biologic fluids when specific extraction procedures are not practicable.

SUMMARY

Techniques of liquid scintillation assay of carbon^{14} have been evaluated for the measurement of plasma and urinary radioactivity, following the administration of histamine-C^{14} to human beings. The only practicable method consisted of the incorporation of water-soluble material into a mixture of p-dioxane, anisole, and 1,2-dimethoxyethane (polyether 611), which also contained two standard scintillation phosphors. Problems of solubility necessitated the dilution of all samples with water, thus reducing the amount of sample which could be assayed. Other advantages of liquid scintillation assay, such as ease of sample preparation and high counting efficiency (58.6 per cent), made this approach worthwhile. Radioactivity equivalent to 0.3 μg histamine base per 100 ml. plasma or 8 μg per liter of urine could be reliably detected. The variable tendency of urine samples to quench scintillation necessitated the addition of an internal standard to each sample in order to establish counting efficiency.

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THE PRESERVATION OF TYPING SERA AT ROOM TEMPERATURE WITH A FIVE PER CENT AQUEOUS SOLUTION OF PHENOL

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IN AREAS where the supply of electricity is scarce, and hospital and blood bank facilities are not easily available, there is obviously a need for typing sera which can withstand deterioration and loss of agglutinating power at room temperature for a certain length of time. Such sera can be stored in the physician's bag, and typing of the patient and prospective donors can be done conveniently at the bedside. If transfusion sets for both donor and recipient are at hand, transfusion may be administered in the home of the patient.

Among the preservatives tried, we have found that a 5 per cent aqueous solution of phenol added to the serum in the proportion of 1 part of the preservative to 10 parts of the serum to be the most effective. The state of preservation lasts for *four* months without noticeable loss of the initial agglutinative potency. In addition, the tendency of the red cells to form rouleaux during the test is considerably reduced so that blood typing can be finished without the aid of a magnifying lens.

MATERIALS AND METHODS

Materials.—1. Separate samples of type A and type B blood from which the anti-B and the anti-A sera, respectively, are prepared.

2. A 5 per cent aqueous solution of phenol, C.P.

3. Type A and type B red cells from donors other than those supplying the sera. Three to 5 drops of blood from a finger-tip wound is placed in a shallow container, such as an empty penicillin vial which is clean and dry. Anticoagulant and normal saline solution are not required. The blood is allowed to clot and the free red cells are used.

4. Coloring solutions. We are using methylene blue, and orange G (National Aniline Division) dissolved to saturation in a 5 per cent aqueous solution of phenol.

6. Ordinary laboratory equipment. Only those instruments to be used for withdrawing blood need be sterile.

Methods.—The following procedure has been found to be the simplest and the one which yields the most consistent results.

1. Withdraw the previously typed blood from the vein, and allow it to clot in a dry container.

2. Separate the liquid portion from the coagulum and centrifugalize to rid the former completely of red cells.

3. Place and measure the amount of serum in a graduated cylinder.

4. Calculate and add the required amount of the phenol solution which is one-tenth of the volume of the serum. The addition is done drop by drop, stirring the solution after each drop to dissipate the cloudiness formed.

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5. Recheck the identity of the isoagglutinin content of the serum with known A and B cells.

6. Determine the agglutinative potency of the serum by counting the number of seconds elapsing from the moment when a drop of serum is mixed with a drop of the opposing cells to the moment when agglutination becomes macroscopically evident. We have arbitrarily set the initial potency of *15 seconds* as the standard of acceptance. Sera with weaker initial potency are rejected. Because too few or too many red cells may delay the early recognition of the agglutinates, it is preferable at the start of the test to place the drops of serum and red cells side by side on the slide without touching each other. Then, sufficient amount of red cells to form a cloudy suspension is brought to the serum by the blunt tip of a toothpick. The mixing is continued till clumped particles are unmistakably visible.

7. Add the coloring solution to the desired intensity, blue for the anti-A, and orange for the anti-B in accordance with usage.

8. Centrifugalize the resulting solution to free it of precipitated matter, store in a convenient container, and label. The serum is then ready for use.

In several instances, one encounters a fresh serum which causes the red cells to group into big rouleaux which are macroscopically difficult to distinguish from agglutinates. To avoid possible confusion, the phenol solution should be added first, before the identity of the isoagglutinins is rechecked.

RESULTS AND DISCUSSION

To test the dependability of the sera prepared with the method described above, samples are included among the permanent contents of our medical kit. They are being used by us and our students almost daily. So far more than one thousand individuals have already been typed. No special precaution has been taken in handling them.

The sera are found to be as reliable as those marketed by government-licensed laboratories in the United States from where most of the typing sera used in the Philippines come. They remain unspoiled at ordinary temperature which in Manila and its suburbs may rise to 35° C. during the months of May and June. They retain their initial potency for *four* months. After this period, there is a gradual decline of strength. Although they can still be used, the time limit for reading the final result should be extended accordingly.

The preservative not only prevents deterioration and loss of potency, but also forestalls the formation of red cells during typing into big rouleaux. No difficulty is thus met even by beginners in recognizing agglutination with the naked eye.

A more direct method of measuring the potency of the sera is adopted and preferred to the cumbersome titration. It enables the examiner to test, and keeps him always aware of, the strength of his sera as he uses them.

SUMMARY

1. A method of preserving typing sera with 5 per cent aqueous solution of phenol has been described. Sera prepared thus keep their initial potency for 4 months without refrigeration and, by preventing the formation of rouleaux, facilitate reading of the result without magnifying lens.

2. Physicians practicing in communities where electricity and blood bank facilities are absent may find this kind of sera of great service to them.

INACTIVATION OF COOMBS REAGENT

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STRICT avoidance of contamination of the Coombs reagent with serum when performing the Coombs test is of the utmost importance in order to avoid a false negative result. According to Boorman and Dodd,[1] one part of globulin in several thousand parts of saline would diminish the activity of the anti-human-globulin serum. Mollison[2] states that one drop of serum diluted 1:5,000 to 1:10,000 will inhibit the reaction of weakly sensitized red cells with a drop of diluted antihuman-globulin serum. To simulate insufficient washing of red cells, Dunsford, Cowen, and Malone[3] added twofold dilutions of AB serum to washed sensitized Rh positive red cells and then added the Coombs reagent; to simulate traces of human serum on the walls of the Coombs pipette, they added twofold dilutions of AB serum to the Coombs reagent and then washed sensitized Rh positive red cells. In both cases they found that one drop of a 1:400 dilution of AB serum (i.e., 0.0001 ml.) was sufficient to inactivate one drop (0.04 ml.) of the Coombs reagent.

The sources of contamination of the Coombs reagent are (1) the saline remaining trapped among the red cells after the third wash (if the red cells are not washed efficiently), (2) the saline used for washing and resuspending the red cells, and (3) the pipette used for transferring the washed red cells and Coombs reagent to the tile. Consequent to this the following steps are recommended: (1) the supernatant serum after the incubation period in the indirect Coombs test should be discarded before the red cells are washed; (2) the red cells must be washed 3 times with large volumes of clean saline—at least 100 times their own volume[1]—and resuspended in clean saline; (3) the clean saline must be obtained from a source which is unlikely to be contaminated with serum, i.e., from a plastic wash bottle filled directly from the stock saline or from a special beaker which is used only for the Coombs test; and (4) a special pipette must be used for transferring the red cell suspension and Coombs reagent to the tile.

In hospital laboratories the indirect Coombs test is very frequently performed as a Coombs compatibility test. Because of the tediousness of the test, especially the repeated centrifuging of the red cells, short cuts may be taken and false negative results obtained with low-titer antibodies.

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The purpose of the present investigation is to re-examine the importance of the above-recommended procedures by noting the effect on the detection of weak antibodies if any one of the above is omitted.

MATERIALS AND METHODS

Procedures.—A preliminary investigation was carried out in the following manner.

1. The indirect Coombs test was performed in duplicate on Rh D positive red cells using incomplete anti-D serum. After incubation at 37° C. for 1½ hours, the supernatant serum of one test only was removed. The red cells of each test were resuspended in a large volume of clean saline, centrifuged, and the supernatant saline poured off into separate clean tubes. The red cells of each test were washed a total of 5 times in a similar fashion, the supernatants in each case being saved.

2. Pipette saline. Clean saline from the stock bottle was poured into a clean tube and one of the pipettes that is in normal everyday use in the blood bank was rinsed out very thoroughly in this saline.

3. Beaker saline. A sufficient amount of the saline remaining in a beaker after half to three-quarters had been discarded when rinsing out the pipettes used in grouping and compatibility tests was poured into a clean tube. The 10 tubes of step 1 and the tubes of steps 2 and 3 were centrifuged to remove all traces of red cells. Double dilutions of the anti-D serum were made commencing at 1:800. Two-tenths milliliter of 25 per cent sulfosalicylic acid was then added to 2 ml. amounts of the saline from each of the 12 tubes, and from the anti-D serum dilutions. The resulting turbidities were measured in a spectrophotometer at 500 mμ. The results are indicated in Table I.

TABLE I. PRELIMINARY INVESTIGATION: TURBIDITIES OF VARIOUS PROTEIN SOLUTIONS

EXPERIMENT	SPECTROPHOTOMETRIC READINGS × 100			
	TEST I	TEST II	SERUM DILUTIONS	
First wash saline	Not done	Not done	1:800	5.5
Second wash saline	2.0	6.75	1:1,600	7.5
Third wash saline	1.25	4.0	1:3,200	4.0
Fourth wash saline	0.75	3.25	1:6,400	2.0
Fifth wash saline	0.25	1.75	1:12,800	1.0
			1:25,600	0.5
Pipette saline	0.75			
Beaker saline	4.5			

The importance of removing the serum prior to washing the red cells in the indirect Coombs test was not examined further, but the third wash saline, in conjunction with the other problems, was investigated more thoroughly as shown below.

Reagents.—

Red cell suspensions: Group O Rh D positive red cells were used as a 50 per cent suspension.

Anti-D serum: Incomplete anti-D serum was used undiluted and in the following dilutions: 1:32, 1:64, 1:128 and 1:256.

Coombs reagent: The antihuman-globulin serum was used at its optimal dilution for the first 5 groups of sensitized cells.

For the sixth group a small volume of this Coombs reagent was used to rinse out a pipette which had been in everyday use for grouping and compatibility tests in the blood bank.

Beaker saline and pipette saline: Beaker and pipette saline solutions were obtained as described previously.

Third wash saline: This was obtained as follows: 2 volumes of the red cell suspension were added to 4 volumes of the undiluted antiserum and incubated at 37° C. for 1½

hours. The supernatant serum was then removed, the cells resuspended in a large volume of clean saline, and centrifuged. The supernatant saline was poured off and the washing repeated twice more. The third wash saline was poured into a tube, centrifuged, and saved.

Methods.—Two volumes of red cell suspensions were added to 4 volumes of the various serum dilutions in tubes (9 cm. long and 0.8 cm. in diameter) and incubated at 37° C. for 1½ hours. This was carried out in sextuplicate. The supernatant sera of the 24 tubes were then discarded and the cells washed 5 times, the supernatant saline of each tube after each wash being removed as completely as possible by means of a pipette attached to a water suction pump.

The cells in each group of 4 tubes were resuspended in (1) clean saline, (2) third wash saline, (3) beaker saline, (4) pipette saline, (5) a mixture of equal parts of third wash saline, beaker saline, and pipette saline and, (6) clean saline.

The Coombs reaction was then carried out on the 6 groups of sensitized cells using the two samples of Coombs reagent as indicated previously. Separate unused pipettes were employed for transferring the sensitized red cells from each tube and the Coombs reagents to the tile.

The scoring of the results was as follows: −ve, no agglutination; ±, just detectable granularity; +, uniform and easily visible granularity; ++, granularity with moderate-sized clumps. The results are indicated in Table II.

TABLE II. EFFECTS OF THIRD WASH SALINE, BEAKER SALINE, ETC., ON THE DETECTION OF WEAK ANTIBODIES IN THE COOMBS TEST

EXPERIMENT	ANTISERUM DILUTIONS			
	1:32	1:64	1:128	1:256
1. Clean saline	++	+	±	−ve
2. Third wash saline	++	+	±	−ve
3. Beaker saline	+	±	−ve	−ve
4. Pipette saline	++	+	±	−ve
5. Mixture of 2, 3, and 4	++	+	±	−ve
6. Clean saline. Pipette rinsed in Coombs reagent	++	+	±	−ve

DISCUSSION

Removal of the supernatant serum after the incubation period and the complete removal of the supernatant saline after each wash with resuspension of the red cells in clean saline is obviously the ideal method, in addition to the 3 washings with large volumes of saline, for the complete removal of all traces of serum or at least extreme dilution. However, removal of the supernatant serum may be omitted by some workers because of the belief that, as the red cells are washed in large volumes of saline, this step is not very important. For the same season the supernatant saline after each wash may just be poured off instead of being completely removed and consequently the red cell resuspension after the third wash is made in the small amount of saline remaining and not in fresh clean saline. If the supernatant serum is not removed prior to washing then obviously the supernatant saline after each wash should be completely removed if the red cells are to be washed efficiently. Pouring off the supernatant saline after each wash is the least efficient of methods for removing the supernatant saline when washing red cells, and, if not preceded by removal of the supernatant serum, sufficient serum may remain to inactivate the Coombs reagent.

In the indirect Coombs test the importance of removing the supernatant serum after the incubation period and the adequacy of 3 washings of the sensitized red cells, especially when combined with the former, is shown by the fact that the quantity of protein remaining in the third wash saline is so dilute (Table I) that it would not interfere with the detection of low-titer antibodies, even if the supernatant saline after each wash is poured away instead of being completely removed (Table II).

Beaker saline becomes increasingly contaminated with serum from the pipettes in everyday use. The experiments show that the concentration may become sufficient to cause a false negative result in the Coombs test with low-titer antibodies.

Inactivation of the Coombs reagent by traces of protein on the wall of the pipette appears to be unfounded. The amount of protein is so little that, even when a very small volume of the Coombs reagent is used to rinse out the pipette, this Coombs reagent is found to be as sensitive as the original in detecting the lowest titer of the antibody. Although the pipette itself is not incriminated, it may still be responsible for false negative results with weak antibodies (1) if it is not thoroughly rinsed out as a routine procedure when in everyday use, or (2) if some beaker saline is inadvertently left inside it when it is used for the Coombs reaction.

The experiments described here confirm the original recommendations. The conclusion to be drawn is that under no circumstances should these procedures be omitted if low-titer antibodies are to be detected.

SUMMARY

The importance of the following procedures in the direct and indirect Coombs test is reinvestigated: (1) removal of the supernatant serum after the incubation period, (2) washing of the red cells 3 times in large volumes of clean saline, and (3) use of a separate beaker and pipette, the Coombs beaker and pipette, containing clean saline for the Coombs reaction.

The results of the experiments carried out confirm that they are important and should not be omitted if false negative results with low-titer antibodies are to be avoided.

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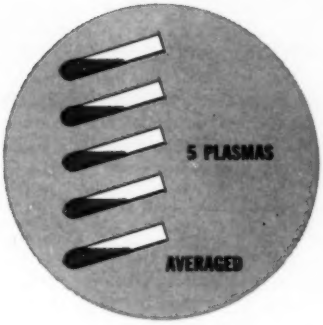

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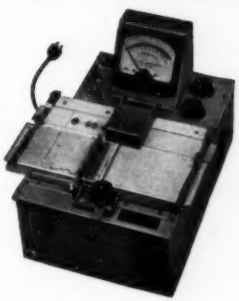

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The Official Publication of the Central Society for Clinical Research

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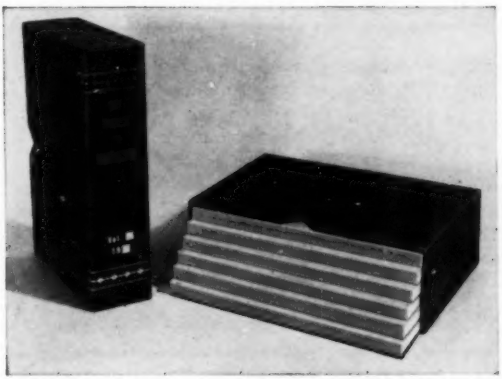
SUMMARY

Several methods of cleaning BOD bottles were compared in order to find methods equivalent to a reference procedure (chromic acid treatment), but more convenient. It was established that a detergent ("7X") gave results quite comparable in reproducibility to the reference method. Other treatments gave less reproducible results.*

* *References Available.*



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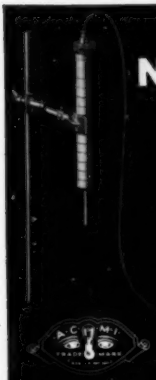

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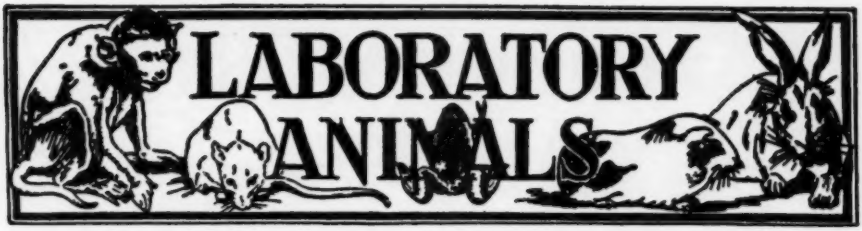

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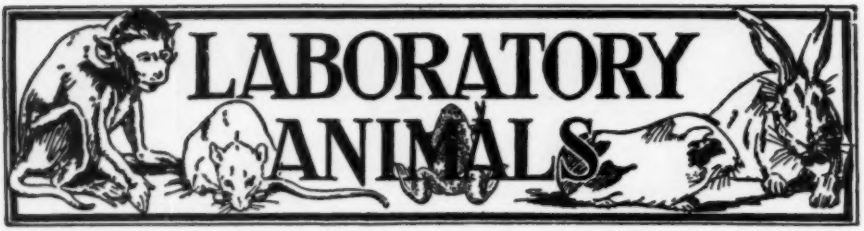

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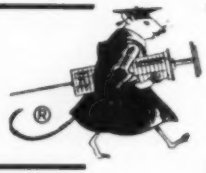

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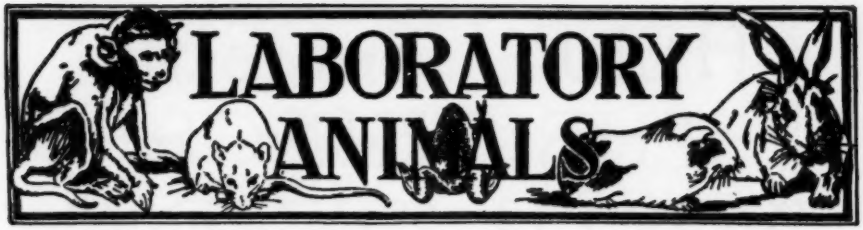

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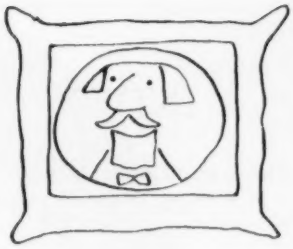

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Biochemical Procedures ---------- 35
Blickman Safety Enclosures ---------- 33
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Cherry Hill Farms (Laboratory Animals) ---------- 37
Clinical Laboratory of Las Vegas ----- 41
Coleman Instruments, Inc. ---------- 20
Cook, Jay E., Importer (Xenopus Laevis Frogs) ---------- 37
Corning Glass Works ---------- 12

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Fisher Scientific ---------- 8

Gradwohl School of Laboratory Technique ---------- 41

Hamilton Laboratory Animals, Inc. --- 38
Hamm Rabbitry, Paul (Rabbits and Guinea Pigs) ---------- 38
Hartman-Leddon Company ---------- 23
Hemlock Hollow Farm (Laboratory Animals) ---------- 39
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Instrumentation Associates ---------- 33

Jones Box Corporation, Jesse ---------- 31

Klett Manufacturing Company -------- 36
Knickerbocker Biosales, Inc. ---------- 41

Lab-Tek Plastics Company ---------- 18
Leitz, Inc., E. ---------- 15
Leo Pharmaceutical Products -------- 19
Linbro Chemical Company ---------- 31

New York Scientific Supply Company (Laboratory Animals) ---------- 38
Nuclear-Chicago Corporation ---------- 2
Nutritional Biochemicals Corporation -- 36

Ortho Pharmaceutical Corporation ---- 7

Paragon C. & C. Company, Inc. ---- 14, 24
Photovolt Corporation ---------- 29

Quivira Specialties Company (Male Frogs) ---------- 39

Rolfsmeyer Company, Dan (Laboratory Animals) ---------- 39

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Scientific Products, Div. of American Hospital Supply Corporation -------- 4
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Steinhilber & Company, Inc., E. G. (Laboratory Animals) ---------- 39
Stocker, J. E. (White Mice, Guinea Pigs and Rabbits) ---------- 37

Taconic Farms, Inc. (Swiss Mice) ---- 38
Technicon Company, The ----- Third Cover
Technicon Company, The ---------- 22
Thomas Company, Arthur H. ---------- 26
Thomas, Publisher, Charles C -------- 6

United States Savings Bonds ---------- 40
Unitron, Instrument Division of United Scientific Company ---------- 21

Volk Radio-Chemical Company ------- 36

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Zeimet Bio-Farms (Guinea Pigs) ----- 38

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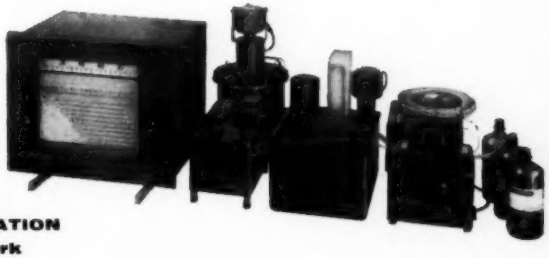

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